THE ROLE OF AUTOPHAGY AND CYSTINE IN THE REGULATION OF AMINO ACID SIGNALING PATHWAYS

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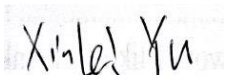
DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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Enjoy!

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Summary

Eukaryotic cells are able to detect amino acid insufficiency and mount adaptive responses. Intracellular amino acid signaling pathways including the mechanistic target of rapamycin (mTORC1) pathway and the integrated stress response (ISR) closely monitor amino acid limitation and imbalance, and integrate stress with cellular processes. The current work demonstrated that amino acid signaling pathways were regulated differentially by the deprivation of total amino acid in cultured myotubes and that of cystine alone in hepatoma cells.

In mouse C2C12 myotubes, it was found that mTORC1 signaling was sustained during total amino acid deprivation. In this context, autophagy was mobilized and it was accountable for the preservation of mTORC1 signaling, which was achieved by the release of amino acids following autophagic proteolysis. Inhibition of autophagy at either early or late stage consistently abolished mTORC1 signaling under amino acid limitation, while this effect could be rescued by amino acid supplementation or inhibition of protein synthesis. Furthermore, results of this study showed that autophagy was the predominant proteolytic process which regulated mTORC1 activity as compared to the ubiquitin-proteasome system. The amino acid profile demonstrated that autophagy maintained cellular amino acid balance during amino acid restriction. In comparison, autophagy fulfills protective functions against the ISR in an amino acid-independent manner. Blockage of autophagy induced the phosphorylation of eIF2α and downstream targets in the ISR system irrespective of amino acid availability. Conversely, the enhancement of autophagy via mTORC1 inhibition suppressed the level of ISR signaling. This study demonstrated an important feedback loop from autophagy to mTORC1 and highlighted the role of autophagy in stress resistance in mouse myotubes, which are both critical for skeletal muscle homeostasis.

However, the response of amino acid signaling pathways follows a different pattern under cystine starvation in the hepatoma HepG2 cells. Deprivation of the unique thiol

amino acid cystine (oxidized form of cysteine) progressively suppressed mTORC1 signaling and acutely induced the ISR. GSH played a protective role against these stresses during cystine limitation, and this was mediated by the release of cysteine through the γ-glutamyl cycle rather than GSH per se. Cystine deprivation elevated GSH export and its ecto-degradation, in an attempt to promote the release of cysteine from GSH. Supplemented GSH preserved mTORC1 signaling and prevented the ISR during cystine limitation, but this protective effect was abrogated when γ-glutamyl transpeptidase (GGT) was inhibited. Inhibition of protein synthesis reduced these stresses, suggesting that the great bulk of cysteine is committed to protein synthesis. Moreover, our results showed that cysteine and GSH cooperate to maintain redox homeostasis and prevent ferroptosis. Altogether, this study demonstrated the critical role of cysteine for mTORC1 signaling and stress prevention, and highlighted the crosstalk between cysteine and GSH in the regulation of amino acid signaling pathways and cell viability.

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List of Abbreviations

4E-BP	eukaryotic initiation factor 4E-binding protein
AA	amino acid
AAR	amino acid response
AARE	amino acid response element
ACTB	actin, β
AMPK	AMP-activated protein kinase
ATF4	activating transcription factor 4
ATF5	activating transcription factor 5
Baf	bafilomycin A1
BCAAs	branched chain amino acids
BODIPY 581/591 C11	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid
BSO	buthionine sulfoximine
C/EBPβ	CCAAT/enhancer binding protein β
CARE	C/EBP-ATF response elements
CASTOR1	cellular arginine sensor for mTORC1
CHOP	C/EBP-homologous protein
Chx	cycloheximide
class I PI3K	class I phosphoinositide-3 kinases
class III PtdIns3K	class III phosphatidylinositol 3-kinase
CMA	chaperone-mediated autophagy
CM-H2DCFDA	5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester
CQ	chloroquine
Deptor	DEP-domain-containing mTOR-interacting protein
DON	6-diazo-5-oxo-L-norleucine
DP	dipeptidase

DPBS	Dulbecco's phosphate buffered saline
EBSS	Earle’s balanced salts solution
ER	endoplasmic reticulum
Fer-1	ferrostatin-1
FIP200	FAK family kinase-interacting protein of 200 kDa
FLCN	folliculin
GADD34	growth arrest and DNA damage-inducible 34
GAP	GTPase-activating protein
GCL	glutamate cysteine ligase
GCN2	general control nonderepressible 2
GEF	guanine nucleotide exchange factor
GGT	γ-glutamyl transpeptidase
GLUD1	glutamate dehydrogenase 1
GOT1	glutamate oxaloacetate transaminase 1
GPT	glutamic pyruvic transaminase
GPxs	glutathione peroxidases
GR	glutathione reductase
GS	GSH synthase
GSH	glutathione
GSHee	glutathione reduced ethyl ester
HRI	heme-regulated inhibitor
HRP	horseradish peroxidase
ISR	integrated stress response
Lac	lactacystin
LC3	microtubule-associated protein 1 light chain 3
MHC	myosin heavy chain
mLST8	mammalian lethal with Sec13 protein 8
mTORC1	mammalian target of rapamycin complex 1

NAC	N-acetyl cysteine
NOX	NADPH oxidase
PAO	phenylarsine oxide
PBS	phosphate-buffered saline
PDK1	3-phosphoinositide dependent protein kinase 1
PE	phosphatidylethanolamine
PERK	protein kinase RNA-like endoplasmic reticulum kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PKB	protein kinase B
PKR	RNA-dependent protein kinase
PRAS40	proline rich AKT substrate 40 kDa
PtdIns3P	phosphatidylinositol 3-phosphate
PTEN	phosphatase and tensin homolog on chromosome ten
PTPs	protein tyrosine phosphatases
Rapa	rapamycin
Raptor	regulatory-associated protein of mTOR
REDD1	DNA damage response 1
Rheb	Ras homolog enriched in brain
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
S6K	ribosomal S6 kinase
SAH	S-adenosyl homocysteine
SAMe	S-adenosyl methionine
SAS	sulfasalazine
SLC38A2	solute carrier family 38, member 2
SLC38A9	solute carrier family 38, member 9

SLC7A1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
SLC7A11	solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11
TBC1D7	Cdc16 Domain Family Member 7
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing 0.1% Tween-20
TFEB	Transcription Factor EB
Tm	tunicamycin
TRIB3	Tribbles 3
TSC1/2	tuberous sclerosis 1/2
ULK1/2	unc-51 like autophagy activating kinase 1/2
UPR	unfolded protein response
UPS	ubiquitin–proteasome system
UVRAG	UV radiation resistance associated
v-ATPase	vacuolar H+-ATPase
Vps34	vacuolar protein sorting 34
WIPI-1	WD repeat domain, phosphoinositide interacting 1

List of Publications

1. Xinlei Yu, Yun Chau Long. Crosstalk between cystine and glutathione is critical for the regulation of amino acid signaling pathways and ferroptosis. Scientific Report.2016; July 18;6:30033.

2. Xinlei Yu, Yun Chau Long, Han-Ming Shen. Differential Regulatory Functions of Three Classes of PI3Ks in Autophagy. Autophagy. 2015; 11(10): 1711-1728.

3. Xinlei Yu, Yun Chau Long. Autophagy modulates amino acid signaling network in myotubes: differential effects on mTORC1 pathway and the integrated stress response. FASEB. 2015; 29 (2): 394-407.

4. Lingying Kong, Daosen Guo, Shiyi Zhou, Xinlei Yu, Guixue Hou, Ronggui Li, Boguang Zhao. Cloning and expression of a toxin gene from Pseudomonas fluorescens GcM5-1A. Arch Microbiol. 2010; 192: 585–593.

Chapter1 General Introduction

Normal cellular metabolism and growth replies on the exogenous supply of nutrients. Amino acids are among the most essential nutrients that constitute the basis of cellular biology. However, the environment is constantly changing, and nutrients are not always available. To cope with the fluctuation in amino acid supply, metazoans have evolved with numerous adaptive responses to modify cellular processes to amino acid availability, and the modulation of signaling pathways is one pivotal means used by the cell to implement the amino acid-driven reprogramming of cellular biology. Two prominent intracellular amino acid signaling pathways are the mammalian target of rapamycin complex 1 (mTORC1) signaling [1, 2] and the integrated stress response system (ISR) [3], both of which closely inspect amino acid signals and control multiple aspects of cellular life.

1. The mTORC1 signaling pathway

mTORC1 signaling is a nutrient- and stress-sensitive pathway. This pathway is highly integrative: a wide spectrum of signal inputs are intertwined by the central kinase complex mTORC1, which branches out into a diversity of anabolic and catabolic processes.

1.1 The constitution of mTORC1

mTOR is a large serine/threonine protein kinase incorporated in two protein complexes, mTORC1 and mTORC2. Compared to mTORC2, mTORC1 is sensitive to rapamycin [4-6], and the regulatory mechanism and downstream targets of mTORC1 pathway are relatively well-characterized. mTORC1 is composed of mTOR, regulatory-associated protein of mTOR (Raptor) [7, 8], mammalian lethal with Sec13 protein 8 (mLST8, or GβL) [9], proline rich AKT substrate 40 kDa (PRAS40) [10] and DEP-domain-containing mTOR-interacting protein (Deptor) [11]. mTOR is a 280 kDa serine/threonine kinase belonging to the phosphoinositide 3-kinase (PI3K)-related

protein kinase, and it is the functional component responsible for the phosphorylative regulation of downstream targets [12]. mTOR consists of five domains: HEAT repeats, FAT domain, FKBP12-rapamycin-binding domain, Kinase domain, and FATC domain [13]. Rapamycin, as the canonical inhibitor of mTOR, forms a complex with FKBP12 and binds to the FKBP12-rapamycin-binding domain of mTOR, leading to the allosteric inhibition of mTOR and the occlusion of its substrate [13, 14]. Raptor functions as a scaffold, which recruits and provides the docking site for substrate proteins [7, 8]. mLST8, also known as GβL, processes unconfirmed regulatory functions toward mTORC1 [9, 15]. PRAS40 is a negative regulator of mTORC1 under the regulation of Akt [10, 16]. Deptor is also present as a constitutive inhibitor of mTOR in this complex [11]. This complex assumes a dynamic conformation which can be modulated by a variety of signals, and it is generally accepted that this complex docks at the lysosome when it is active [17-19].

1.2 Regulation of mTORC1 by multiple signals

mTORC1 signaling is sensitive to a wide variety of signals which represent nutritional and stress conditions. These signals are transduced via specific routes and converge on the central hub mTORC1. Among these signals, growth factors, amino acids, energy status and oxygen levels are the canonical regulators of mTORC1 [20]. The typical signaling transduction pathways for these inputs and the major downstream targets of mTORC1 are depicted in Fig 1.

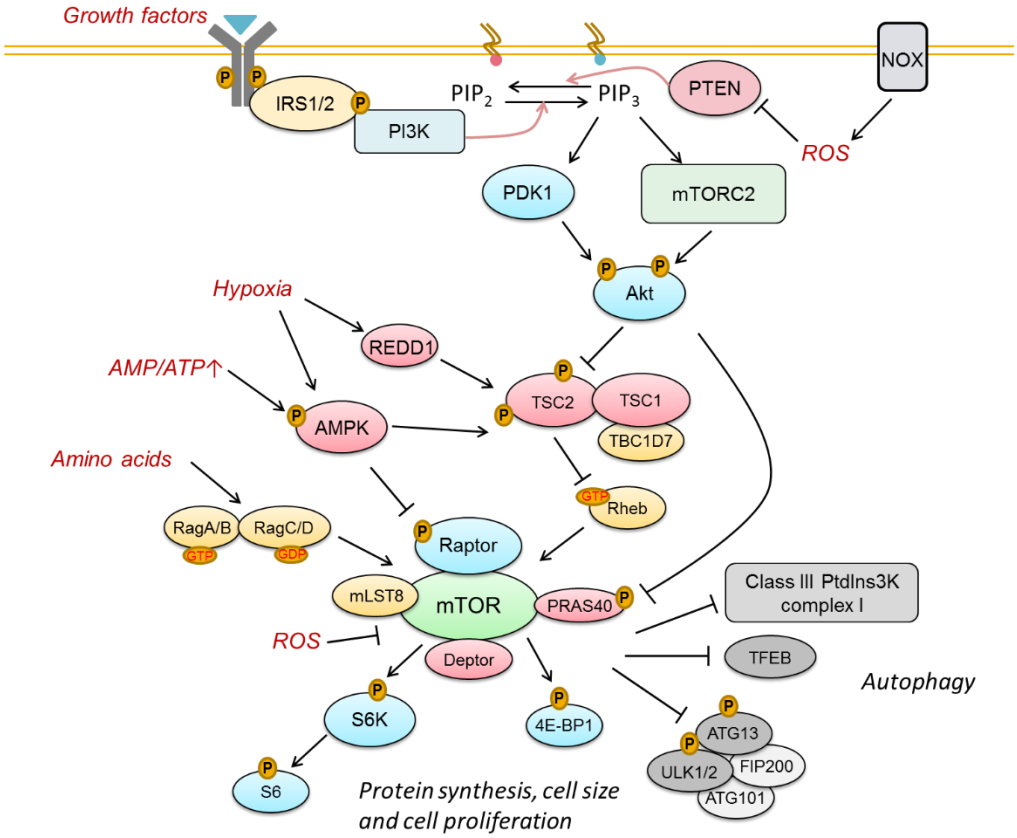


Fig 1. Major upstream inputs, their regulatory pathways, and the main downstream targets in the mTORC1 network. Growth factors stimulate mTORC1 mainly through PI3K-Akt signaling, in which Akt activates mTORC1 via TSC1/2-Rheb and PRAS40. Hypoxia inhibits mTORC1 through REDD1 and AMPK, both of which transduce the signal through TSC1/2, while AMPK acts additionally on Raptor. The increase in cytosolic AMP/ATP ratio, as an indicator of energy stress, activates AMPK, which then suppresses mTORC1 activity. Amino acids are the prerequisite for mTORC1 activity, and most of their signaling cascades converge on Rag complex, which is a determinant of mTORC1 localization and activity. The redox regulation of mTORC1 pathway remains controversial. Tentatively, it is believed that endogenous ROS generated by NOX serve as secondary messengers that activate mTORC1 pathway in part through inhibiting PTEN; in comparison, exogenous high dose of ROS have inhibitory effects on mTORC1 via unestablished means. Two main downstream anabolic signaling axes in mTORC1 pathway are S6K-S6 and 4E-BP1, which control protein synthesis, cell growth and proliferation. mTORC1 suppresses autophagy via multiple nodes, including ULK complex, TFEB and ATG14-containing class III PtdIns3K complex.

Growth factors

Growth factors such as insulin bind to their cognate receptors on the plasma membrane and pass down the signal to mTORC1 mainly via the PI3K-Akt axis. Receptor ligation leads to the activation of the class I phosphoinositide-3 kinases (class I PI3K), which converts phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) [21], a process negatively regulated by phosphatase and tensin homolog on chromosome ten (PTEN) [22]. Concentrated PIP_3 on the inner leaflet of the plasma membrane is required for the recruitment of 3-phosphoinositide dependent protein kinase 1 (PDK1) and Akt, which is also called protein kinase B (PKB) [23]. Subsequently, Akt becomes activated through the phosphorylation by PDK1 and mTORC2 at Ser 308 and Thr 473, respectively [23, 24]. Akt phosphorylates TSC2 (tuberous sclerosis 2) and thereby inactivates the TSC complex composed of TSC1, TSC2, and Tre2-Bub2-Cdc16 Domain Family Member 7 (TBC1D7), which harbors the GTPase-activating protein (GAP) activity towards Ras homolog enriched in brain (Rheb) [25]. When Rheb is switched to the GTP bound state, it activates mTORC1 [25, 26]. In addition to TSC complex-Rheb axis, Akt can directly activate mTORC1 by phosphorylating PRAS40, a negative regulator of mTOR [27].

Amino acids

The presence of amino acids is the prerequisite for mTORC1 activity. mTORC1 can recognize a panel of amino acids, and as so far identified, they mainly include L-leucine [1, 2, 28-31], L-tyrosine [28], L-phenylalanine [28], and L-arginine [1, 31, 32], which are essential amino acids, as well as L-glutamine [33-36], which is an energy fuel and a nitrogen donor. As to the mechanisms by which amino acids regulate mTORC1, a number of models have been proposed [18, 19, 30, 33, 35, 37-39] (the generic and amino acid-specific regulatory mechanisms for mTORC1 are shown in Fig 2). The well-accepted canonical model was proposed by *D.M. Sabatini* group, and in this model, amino acids are believed to regulate mTORC1 through controlling its lysosomal

localization via the core vacuolar H^+-ATPase (v-ATPase)-Regulator-Rag machinery [18, 19, 30]. Specifically, amino acids, which flux into the lysosome after uptake, regulate v-ATPase and lead to a change in its assembly at the lysosomal membrane [18, 40]. Consequently, the interaction of v-ATPase with the Ragulator complex is disrupted, leading to the activation of Ragulator [18]. Ragulator, as a guanine nucleotide exchange factor (GEF), promotes the loading of Rag A/B with GTP [41]. In the meanwhile, folliculin (FLCN) acts as a GAP for Rag C/D and promotes the binding of Rag C/D to GDP [42]. Therefore, the Rag complex is converted to an active state, which consists of Rag A/B-GTP and Rag C/D-GDP. Conversely, in the absence of amino acids, GATOR1 is active and imposes its GAP activity towards RagA/B, inhibiting Rag activity [43, 44]. In the presence of amino acids, mTORC1 is recruited by the active Rag complex and translocates to the lysosome where it encounters Rheb and acquires its full kinase activity [17-19, 30]. In the general scheme of mTORC1 regulation, Rheb and Rag complex, which respond to growth factor and amino acid signaling respectively, act as the main switches for mTORC1 activity [19, 25, 26, 30].

With regard to the amino acid-specific sensing mechanisms, a set of candidate amino acid sensors for mTORC1 have been identified (Fig 2). For instance, solute carrier family 38, member 9 (SLC38A9) and cellular arginine sensor for mTORC1 (CASTOR1) were discovered as plausible arginine sensors [32, 45-47], and Sestrin2 was demonstrated to be a leucine sensor [48]. SLC38A9 is a lysosomal transmembrane protein, which has high affinity for arginine, and after binding to arginine, this protein enhances the GEF activity of Ragulator, which converts Rag A/B to the favorable nucleotide-binding state for mTORC1 activation [32, 45, 47]. CASTOR1 and Sestrin2 both negatively regulate GATOR2, which is an inhibitor for GATOR1 [46, 48]. The binding of arginine and leucine to CASTOR1 and Sestrin2 respectively lead to the activation of the Rag complex and therefore that of mTORC1 [46, 48]. However, controversies remain over the amino acid sensing scheme. For instance, some existing

evidence question the requirement of Rag or Ragulator for the activation of mTORC1 by amino acids [33, 49], and leucine and arginine were shown to have no effect on the lysosomal localization of mTORC1 [49, 50]. The precise mechanism of how mTORC1 senses and responds to amino acids still remains to be defined, and the list of amino acids that stimulate mTORC1 may go well beyond the ones so far recognized.

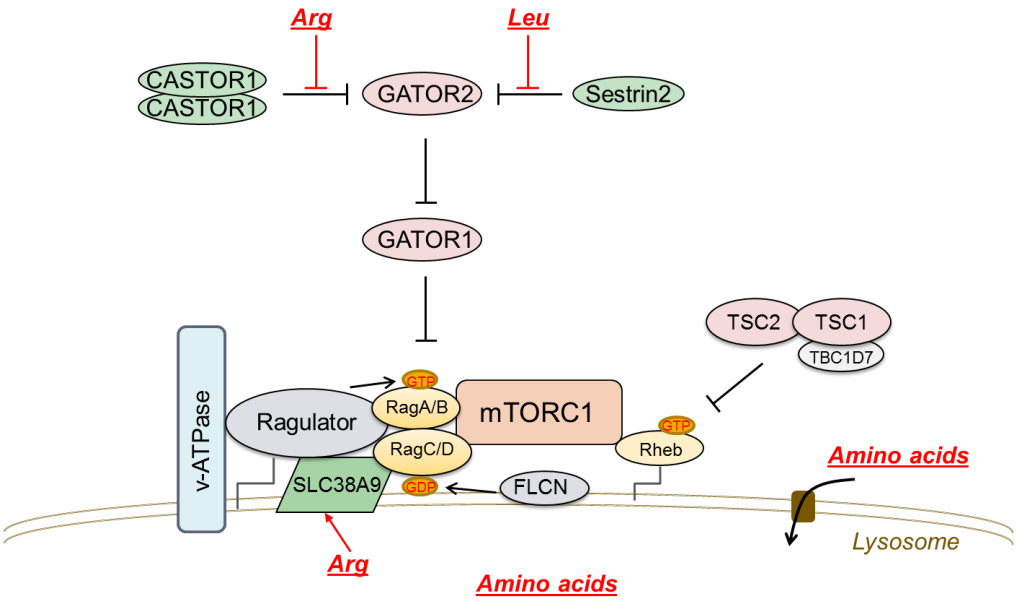


Fig 2. The generic and amino acid-specific sensing mechanisms for mTORC1. Total amino acids are acknowledged to stimulate mTORC1 through the core complex, which is composed of v-ATPase-Ragulator-Rag GTPase. SLC38A9 and CASTOR1 are identified to be arginine sensors, which regulate mTORC1 through Ragulator and GATOR2-GATOR1, respectively. Sestrin2 is plausible leucine sensor, which transmits the signal to mTORC1 through GATOR2-GATOR1. The signals from amino acids converge on Rag complex, which recruits mTORC1 onto the lysosome and activates this complex.

Energy status, hypoxia and redox

In addition to growth factors and amino acids, energy status, hypoxia and redox are also modulators of mTORC1 activity (Fig 1). Energy stress as accompanied by an increase in the cytosolic AMP/ATP ratio, which is sensed by AMP-activated protein kinase (AMPK) [51]. Activated AMPK negatively regulates mTORC1 by phosphorylating TSC2 [52] and Raptor [53], signalling the shortage in energy supply to mTORC1 and decelerating anabolic process. Hypoxia suppresses mTORC1 via the nodes of AMPK and DNA damage response 1 (REDD1), and the latter regulates mTORC1 by promoting the integrity of TSC complex [54]. The redox modulation of mTORC1 has been a controversial topic, and the effect of reactive oxygen species (ROS) on mTORC1 seems to be dose- and context-dependent. Endogenous ROS generated by NADPH oxidase (NOX) may act as positive messengers in receptor tyrosine kinase (RTK) signaling which activate mTORC1 [55, 56], whereas exogenously derived high doses of ROS are insults that suppress mTORC1 activity [57-59]. Intriguingly, it was reported that a potent oxidizing reagent phenylarsine oxide (PAO) dramatically stimulated mTORC1 signaling activity [60, 61], and it may directly act on mTORC1 [60]. Based on these observations, mTORC1 is likely to be susceptible to direct redox modulation on its own, while oxidative stress can also intervene with signaling transduction upstream of mTORC1.

1.3 Downstream of mTORC1

mTORC1 pathway is a versatile signaling network which has ramifications into almost every aspect of cellular biology. Both anabolic and catabolic processes are tightly controlled by this central kinase. In terms of anabolism, protein synthesis is the prototypical energy-consuming anabolic process targeted by mTORC1, along with other metabolic processes such as lipid synthesis, nucleic acid biosynthesis, mitochondrial oxidative function and biogenesis [20, 62]. In concert with anabolic regulation, mTORC1 tightly controls proteolytic degradation including autophagy [63,

64] and the ubiquitin–proteasome system (UPS) [65], and the former represents the canonical degradation process under the control of mTORC1.

S6K and 4E-BP1, two major nodes downstream of mTORC1

Two classical downstream branches in the mTORC1 pathway are ribosomal S6 kinase (S6K)-S6 and eukaryotic initiation factor 4E (eIF4E)–binding protein (4E-BP) axes (Fig 1). Mammalian cells have two S6K proteins, S6K1 and S6K2 [66-68]. p70S6K is the cytosolic isoform of S6K1 whose signaling mechanism is well-characterised. mTORC1 phosphorylates p70S6K at multiple residues including the key modification site T389, leading to the activation of this kinase [69-71]. S6K in turn phosphorylates ribosomal protein S6 as well as other components in the translation apparatus [72, 73]. Although still controversial, it is accepted that phosphorylated S6 selectively enhances the translation of 5'-terminal oligopolypirymidine (5'-TOP)-containing mRNAs that encode ribosomal proteins, elongation factors and other regulatory factors in the protein translation machinery, resulting in the augmentation of the translational capacity [74-76]. The other mTORC1 substrate 4E-BP is a direct binding partner of translation initiation factor. In the unphosphorylated state, 4E-BP binds to eIF4E and hinders its interaction with eIF4G, impeding cap-dependent translation initiation [77]. mTORC1 releases eIF4E from this inhibitory complex through phosphorylating 4E-BP and thereby promotes cap-dependent translation [77-79]. As two ultimate phenotypes, cell size [80] and cell proliferation [81, 82] are also under the strict control of mTORC1 via S6K, 4E-BP1 and other effectors in keeping with endogenous and exogenous conditions.

Autophagy

mTORC1 impinges on multiple points along the autophagic pathway to tightly control this catabolic process (the major regulatory nodes are shown in Fig 1, and the detailed mechanisms are presented in Chapter 2 and Fig 5). At the onset of autophagy, mTORC1 curbs the initiation of autophagy through unc-51 like autophagy activating kinase 1/2

(ULK1/2)-ATG13-FAK family kinase-interacting protein of 200 kDa (FIP200)-ATG101 complex [83-85]. When intracellular nutrients are abundant, mTORC1 phosphorylates ULK1/2 and ATG13, and thereby destabilizes and inactivates this autophagy-initiating complex. Under nutrient deprivation or other stresses, the ULK1/2-ATG13-FIP200-ATG101 complex is released from the inhibitory control of mTORC1 and commences the signalling and biochemical events in autophagy [83-85]. Additionally, mTORC1 activity is also coordinated with autophagic activity through Vps34, the key phosphatidylinositol 3-kinase (PtdIns3K) in the autophagic pathway [86]. Moreover, mTORC1 also regulates the generation of autophagic and lysosomal machinery, for instance, through the phosphorylation and inhibition of Transcription Factor EB (TFEB) [64, 87-89]. The multipronged regulation of autophagy is a prominent example of the exquisite actions of mTORC1, which puts a brake on catabolism when nutrient is abundant while rapidly mobilizes internal nutrient store under adverse conditions.

1.4 mTORC1 in health and diseases

The last decades witnessed a wave of growing interest and effort in the research field of mTORC1. Along with the increasingly clear picture elucidating the mechanisms underlying the regulation of mTORC1 by various signals, the role of mTORC1 in organ and whole body function and pathogenesis of diseases is becoming better appreciated. The diverse spectrum of diseases resulting from mTORC1 dysfunction are virtually a reflection of the divergent regulatory functions of this complex in different aspects of cellular biology. Hyperactivity of mTORC1 is heavily involved in cancer, which is not only due to the pro-anabolic effect of mTORC1, but also related to the suppression of the cellular housekeeping process of autophagy [90]. Dysregulated mTORC1 activity also contributes to metabolic disorders represented by obesity and diabetes through its regulation of metabolic functions [90, 91]. In addition, mTORC1 controls cell mass in part through its contribution to protein synthesis, for which one typical example is its

delicate regulation of skeletal muscle mass. Abnormal activity of mTORC1 at the two extremes including hyperactivity and underactivity leads to hypertrophy [92] and atrophy [93], respectively. Another intriguing physiological relevance of mTORC1 is its involvement in aging. Targeting mTORC1 by genetic or pharmaceutical means showed potential to promote longevity in different model organisms under experimental settings [91]. Development of effective therapeutic approaches for the treatment of mTORC1-implicated diseases will definitely be driven by the increasing understanding of this complex at the mechanistic level.

2. The ISR pathway

2.1 The four stress sentinels in the ISR

The ISR is another highly integrative signaling network that orchestrates diverse cellular processes in accordance with adverse conditions. This system has specific upstream kinases responsible for inspecting distinct stresses at the top tier, which transduce the signal to the central node eIF2α. The ISR has a comprehensive impact on not only the entire proteome but also the whole transcriptome.

Four highly specialized stress-sensing kinases reside upstream of eIF2α in the ISR network: general control nonderepressible 2 (GCN2), which scrutinizes the limitation of amino acids within the cell and accounts for the initiation of the amino acid response (AAR); the double-stranded RNA–activated protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK), which locates at the ER and mounts the unfolded protein response (UPR) in response to ER stress; RNA-dependent protein kinase (PKR), which is induced by double-stranded RNA (dsRNA) and participates in the inflammatory response to viral infection; heme-regulated inhibitor (HRI), which is mainly stimulated by heme deficiency [94]. After being activated by respective stimuli, these kinases phosphorylate eIF2α at Ser-51, which in turn suppresses general protein translation and passes the stress signal down to downstream targets (The core signaling routes in the ISR system are depicted in Fig 3).

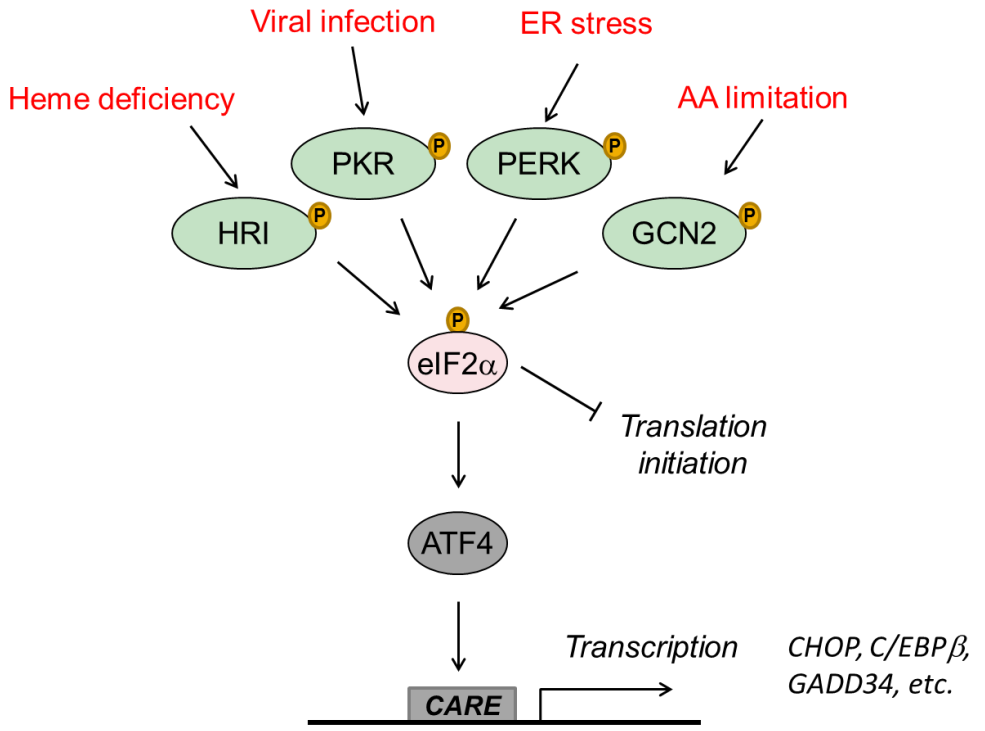


Fig 3. The ISR system. Four upstream kinases specifically sense four types of stresses and phosphorylate eIF2α upon perturbation. eIF2α attenuates translation initiation while inducing the translation of ATF4, which mediates the CARE element-driven transcription of target genes.

2.2 The eIF2α-mediated global effect on translation

eIF2α is a subunit of the heteromeric translation initiation factor eIF2. Functional eIF2, in which eIF2α is linked to GTP, from a complex with Met-tRNA$_i^{Met}$ [95]. This ternary complex then binds to the 40S ribosomal subunit, which recognizes the start codon at the mRNA [95]. After start codon recognition, eIF2-GTP is hydrolyzed to eIF2-GDP, leading to the inactivation of this transcription initiator. The recycling of eIF2-GDP to eIF2-GTP, catalyzed by the GEF eIF2B, is required for launching another round of the translation initiation process. However, when eIF2α is phosphorylated at Ser51, the identity of eIF2 is converted from a substrate to an inhibitor of eIF2B, halting the recycling of eIF2 [95]. The direct alteration in the activity of translation initiation factor rapidly and potently attenuates global protein translation, stalling time for adaptive response and reducing the consumption of energy and materials during stress.

Nevertheless, in parallel with the overall translation inhibition, several transcription factors are paradoxically upregulated by phospho-eIF2α, including activating transcription factor 4 (ATF4) [96], growth arrest and DNA damage-inducible 34 (GADD34) [97], and activating transcription factor 5 (ATF5) [98]. These transcription factors elicit a diverse range of adaptive responses according to the stress conditions. Among them, the classical eIF2α downstream effector ATF4 differentially regulates the transcription of target genes via binding to their consensus C/EBP-ATF response elements (CARE), and regulates a variety of cellular functions including protein synthesis, amino acid metabolism, oxidative stress response, cell growth and cell death [99-101].

2.3 The AAR branch in the ISR system

AAR is an important branch in the ISR network. The limitation of amino acids is sensed by GCN2, and through the GCN2-eIF2α-ATF4 axis, it imposes comprehensive transcriptional and translational alterations in the cell [102]. The specialized amino acid sensor GCN2 monitors the shortage of intracellular amino acids by virtue of binding to the uncharged tRNA. To be exact, under conditions of amino acid scarcity, the accumulated cognate uncharged tRNAs bind to the histidyl-tRNA synthetase-like (HisRS) domain in GCN2 protein, eliciting a conformational change in this kinase [3, 103-106]. Subsequently, a series of events including dimerization [107] and autophosphorylation (e.g. Thr 882/887 in yeast, and Thr 899 in mammals) [108] take place, conferring full kinase activity to GCN2. Activated GCN2 in turn phosphorylates eIF2α at Ser-51 [109], leading to a suppression of overall protein translation coupled with a selective upregulation of transcription factors such as ATF4 [102]. As mentioned above, ATF4 recognized the CARE consensus motif in its target genes, and in the context of AAR, this conserved sequence is termed the amino acid response element (AARE) [102]. Canonical examples of these AARE-containing genes include asparagine synthetase (ASAN), C/EBP-homologous protein (CHOP),

CCAAT/enhancer binding protein β (C/EBPβ), GADD34, cationic amino acid transporter 1 (CAT1), and Tribbles 3 (TRIB3). Their differential expression in response to amino acid limitation reprograms an extensive spectrum of cellular processes including protein synthesis, amino acid metabolism, cellular redox, cell viability and proliferation [102]. Therefore, the activation of AAR under amino acid limitation or imbalance has a vast and significant impact on cellular biology beyond amino acid metabolism.

2.4 The pathological relevance of the ISR

The ISR pathway functions as a homeostat to adjust cellular and systematic processes in tune with stress contexts, while the dysfunction of this adaptive response system is implicated in a spectrum of diseases. *In vivo*, deletion of GCN2 impaired the ability of the body to adapt to amino acid deprivation, and these GCN2 knockout mice displayed dysregulated liver lipid metabolism [110], loss of skeletal muscle mass [111], and mortality [111, 112] under leucine deprivation. PERK is critical for pancreatic functions, since the loss of PERK either during embryogenesis or after birth resulted in the degeneration of pancreatic islets and deficit in insulin secretion [113-115]. eIF2α is also a key regulator of glucose homeostasis as well as lipid metabolism. Mice with homozygous dominant negative mutant of eIF2α (S51A), which could no longer be phosphorylated under stresses, displayed hypoglycaemia and died one day after birth [116]. eIF2α $^{S51A/+}$ mice were viable and phenotypically normal when fed with standard diet, but exhibited metabolic abnormalities such as hyperlipidemia and glucose intolerance when fed on high fat diet [117]. The downstream effector ATF4 plays a myriad of important biological roles not only under stress but also at basal conditions. ATF4 null mice showed hypoglycaemia, hypoinsulinemia [118], dysregulated lipid metabolism [119], and lens defect [120]. Furthermore, the ISR pathway is closely related to cancer because of its role in mediating stress resistance and survival. Multiple key proteins in this network including GCN2 [121], PERK [122], eIF2α [123], and

ATF4 [124] have been linked to carcinogenesis and drug resistance. Taken together, the ISR pathway holds great potential as a target for treating metabolic diseases, cancer and other pathological conditions.

3. Concluding remarks and the introduction to my studies

mTORC1 signaling and the ISR are the principal intracellular amino acid signaling pathways which integrate nutritional status with a multitude of cellular processes. Apart from the vital signals of amino acids, these two pathways are also susceptible to the modulation of various stresses. In response to external and internal cues, they control global protein synthesis, metabolism and cellular hemostasis, which can be translated at the physiological level into organ and whole body function. Tremendous progress has been made towards the elucidation of these two pathways; however, there are still some open questions and elusive points in this area. One point worthy of further investigation is their context- and stress-specific response, which is relevant in certain physiological and pathological scenarios.

The present studies aimed to reveal how the cell responds to amino acid limitation, including both total amino acid starvation (in the first study) and cystine deprivation (in the second study), through the mTORC1 and ISR pathways. The former circumstance represents the condition of general nutritional deprivation and the post-absorptive state, in which the amino acid supply becomes scarce; the latter mimics the cysteine-deficient state under certain malnutritional and pathological conditions. In the first study, skeletal muscle cells (mouse myotubes) were used as a model because skeletal muscle is metabolically flexible and accounts for a large proportion of inter-organ nutrient supply under nutrient-limited conditions. In this study, I found that upon amino acid starvation, mouse myotubes managed to sustain mTORC1 signaling, and this was attributed to the intrinsic amino acid provision by autophagic degradation; the ISR system, though, was activated under autophagic failure independent of amino acids. In the second study, the hepatoma cell line HepG2 was utilized as a model given

that liver cancer cells reply predominantly on extracellular cystine as a source of cysteine. The results demonstrated that cystine limitation suppressed mTORC1 while inducing the ISR, and GSH functioned as a protective force under this circumstance which partially prevented the alteration of cell signaling and maintained cell viability. Thus, these studies illustrated the specificity in the cell signaling response to disparate nutrient stresses in different cellular contexts, and demonstrated how the lack of general amino acids or single amino acid alters cell signaling and related processes.

Chapter 2 Autophagy differentially regulates mTORC1 and the integrated stress response in cultured myotubes

1. Introduction

1.1 Autophagy

The term autophagy encompasses three types of degradation processes that terminate in the lysosome: macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy. While macroautphagy is a well-characterized sequential membrane trafficking process, CMA or microautophagy is more of a selective proteolytic process that directly targets substrates to the lysosome for degradation. Since macroautophagy is the focus of this study, hereafter macroautophagy is generally referred to as autophagy if not otherwise specified. During autophagy, proteins and organelles are sequestered by progressively evolved double-membrane structures termed autophagosomes and then delivered to the lysosome for degradation [125]. In this process, while cellular components can be degraded into smaller biomolecules for biosynthetic or bioenergetic usage [126], misfolded proteins, aggregates, and damaged organelles are preferentially eliminated to obviate cytotoxicity [127]. In this regard, autophagy serves both nutrient-recycling and housekeeping functions. A wide spectrum of stresses can induce autophagy, such as nutrient limitation [128], oxidative stress [129], ER stress [130], and virus infection [131]. Prolonged autophagy can culminate in either cell survival or cell death, depending on the severity and nature of stress and cellular context [132].

1.1.1 The process of autophagy

Autophagy is a highly ordered and tightly regulated degradation process composed of a series of signaling and membrane evolution events. The whole process of autophagy can be divided into five stages: initiation, nucleation, expansion, maturation, and degradation (The major regulatory machinery and membrane evolving events in

autophagy are shown in Fig 4). The progression of autophagy is driven by the autophagic machinery which is composed of several complexes, including the ULK complex, the ATG14 containing-class III phosphatidylinositol 3-kinase (PtdIns3K) complex, the ATG9-ATG2-WD repeat domain, phosphoinositide interacting 1 (WIPI-1)/Atg18 complex, and two ubiquitin-like conjugation systems [125]. Upon autophagy induction, ULK1/2 and ATG13 become dephosphorylated and form an active complex together with FIP200 and ATG101 [83-85]. The active ULK complex is then recruited to the nucleation site for membrane formation, which is called the omegasome in mammalian cells [133-135]. Following ULK relocation, the class III PtdIns3K complex which is composed of vacuolar protein sorting 34 (Vps34)-Beclin 1-Vps15-ATG14 (class III PtdIns3K complex I) translocates to the ULK-enriched site and generates phosphatidylinositol 3-phosphate (PtdIns3P) at the nucleation site [133-135]. PtdIns3P is a key messenger triggering the recruitment and assembly of the protein apparatus for the subsequent membrane curvature and remodeling [136, 137]. These favorable conditions enable the formation of a primary double membrane, cup-like structure called the phagophore at the nucleation site [138]. The elongation of phagophore and its maturation into autophagosome proceed with the aid of two ubiquitin-like conjugation systems, including ATG5-ATG12-ATG16 system and the microtubule-associated protein 1 light chain 3 (LC3) lipidation system. The first conjugation system links ATG12 with ATG5, which then forms a complex with ATG16, while the second conjugation system that is comprised of ATG4, LC3 (mammalian homologue of yeast Atg8), ATG7 and ATG3 tags LC3 with phosphatidylethanolamine (PE) [139]. The PE-linked LC3 protein accumulates on phagophore and autophagosomal membranes, creating a positive signal for membrane expansion, curvature and closure [140], as well as the specific targeting of autophagy substrate [141]. The expansion of autophagic membrane also requires ATG9-ATG2-WIPI-1/ATG18 complex, which likely serves as a recruiter for membrane precursors to build up on the growing phagophore [142, 143]. At the final stage, the

autophagosome is fused with the lysosome to form the autolysosome, in which the engulfed substrates are degraded by lysosomal enzymes [127]. However, autophagy does not end with substrate degradation, but is followed by the reformation of tubular structure on the autolysosome, which finally matures into the lysosome [144]. The reformation and scission of *de novo*-generated lysosomes are dependent on the activity of mTORC1 and involve Vps34-UV radiation resistance associated (UVRAG) [144, 145].

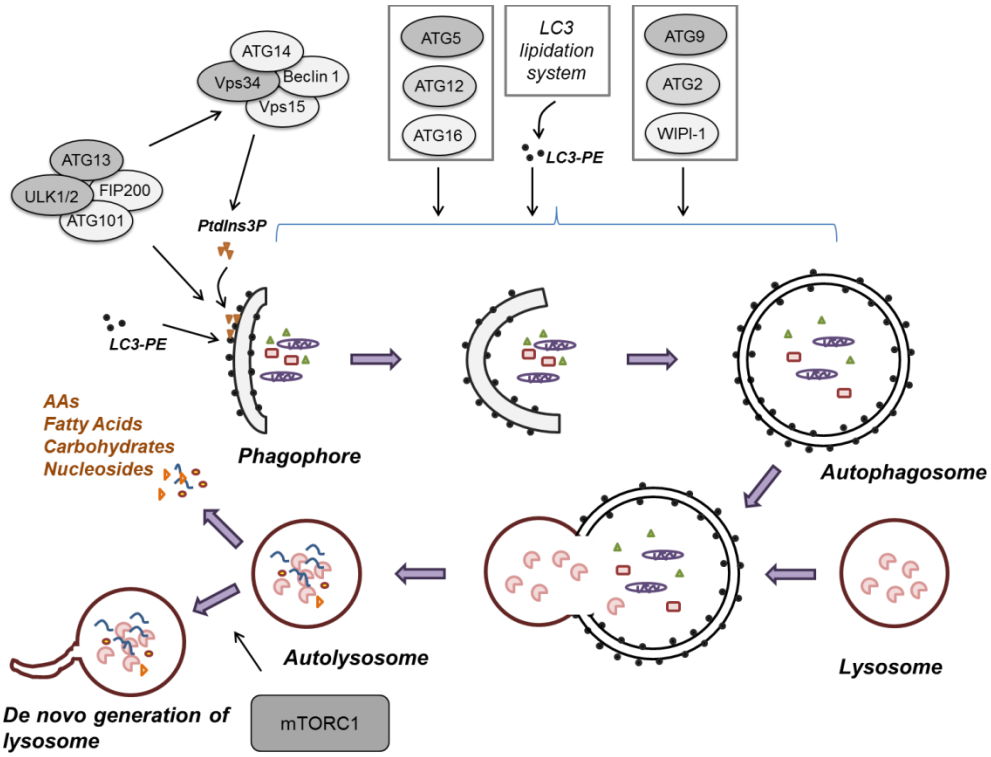


Fig 4. The process of autophagy and its main regulatory machinery. Autophagy is a membrane-trafficking process, in which phagophore evolves into an autophagosome and finally, an autolysosome. After substrate degradation in the autolysosome, lysosome is regenerated on the autolysosomal membrane. An array of molecules are involved in the entire process of autophagy, which mainly include the ULK1/2 complex, the ATG14-containing class III PtdIns3K complex, ATG5-ATG12-ATG16 conjugation system, LC3, LC3 lipidation system, ATG9-ATG2-WIPI-1 and mTORC1. Their engagement in different stages of autophagy is indicated in the diagram.

1.1.2 Regulation of autophagy by mTORC1 and the ISR

Autophagy is a programmed process that involves a number of regulators, such as mTORC1, AMPK, Vps34, eIF2α, and ATF4 [146]. Among these regulators, mTORC1 is the classical controller that determines the onset, progression and activity of autophagy in a nutrient-sensitive way [147]. As another stress-response system, the ISR also heavily engages in this degradation process in the event of various stresses [123, 128, 148-153]. The primary mechanisms by which mTORC1 and the ISR regulate autophagy are shown in Fig 5.

mTORC1 places multiple brakes on the process of autophagy. Active mTORC1 phosphorylates ULK1/2 and ATG13, and these modifications destabilize this complex and prevent it from commencing the autophagic process [83-85, 154]. Under starvation or pharmaceutical inhibition of mTORC1, ULK1/2 and ATG14 are no longer fettered by mTORC1 and become dephosphorylated, so that ULK1/2 resumes its kinase activity towards ATG13 and FIP200 and converts the complex into an active initiator of autophagy [83-85, 154-156]. Besides, ULK1/2 also positively regulates autophagy through activating the ATG14-containing class III PtdIns3K complex [157]. Furthermore, mTORC1 strictly coordinates its activity with that of the ATG14-containing class III PtdIns3K complex which is critical for both initiation and progression of autophagy, through the phosphorylation of ATG14 and inhibition of Vps34 kinase activity [86]. At the end stage of autophagy, mTORC1 promotes the regeneration and precise scission of newly formed lysosomes, and the latter is mediated through the UVRAG-containing class III PtdIns3K complex (class III PtdIns3K complex II) [144, 145]. At the transcriptional level, mTORC1 regulates the synthesis of autophagic machinery and lysosomal proteins via TFEB. Under normal conditions, active mTORC1 phosphorylates TFEB and retards its nuclear translocation; conversely, mTORC1 inhibition under stresses disenthralls TFEB as a transcription

factor, which transfers to the nucleus and promotes the expression of lysosomal and autophagic genes [87, 88, 158].

In contrast to the well-characterized regulation of autophagy by mTORC1, the mechanism by which the ISR regulates autophagy remains largely unknown. A body of evidence demonstrated the involvement of this pathway in the induction and progression of autophagy [123, 148-152, 159], whereas defects in the ISR, especially the loss-of-function in eIF2α phosphorylation, significantly impaired the ability of the cell to mobilize autophagy in the face of stresses [148, 150]. As to the plausible mechanisms, transcriptional control of the key genes in the autophagic system, such as *MAP1LC3B*, *SQSTM1*, *BECN1*, and several *ATG* genes, is one means by which this stress response system exerts control over this degradation pathway [151, 159, 160]. Although more needs to be unraveled in the regulatory scheme, it is highly likely that the ISR system holds an equally important role as mTORC1 in the modulation of autophagy. These two nutrient- and stress-sensitive pathways stringently control this self-degradation system, which allows the cell to promptly adapt to altered environment and recover from stress-induced dyshomeostasis.

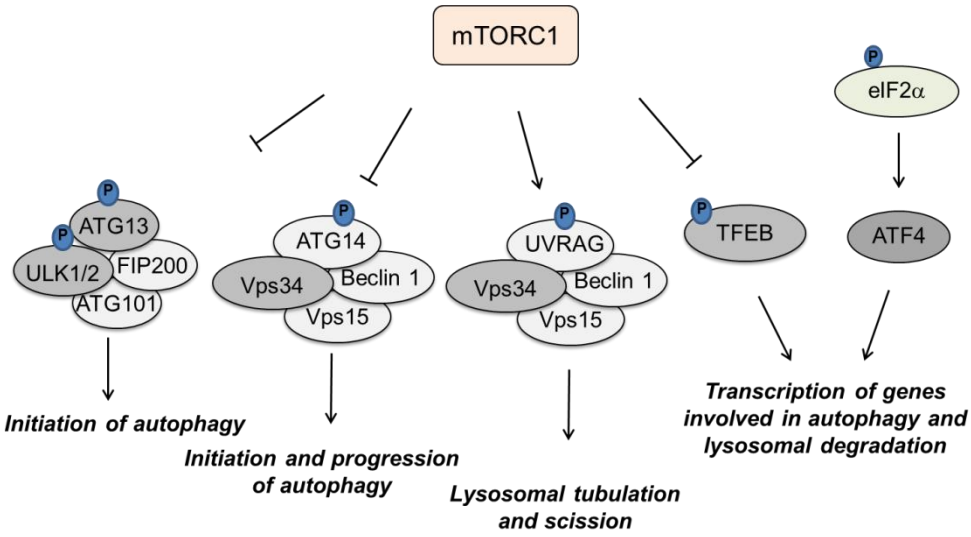


Fig 5. Regulation of autophagy by mTORC1 and the ISR. mTORC1 imposes multipronged regulation on autophagy. It inhibits the initiation of autophagy through the ULK1/2 complex, suppresses its initiation and progression through the ATG14-containing class III PtdIns3K complex, promotes the reformation of lysosomal tubules and its precise scission in part through the UVRAG-containing class III PtdIns3K complex, and regulates the transcription of genes encoding the autophagic and lysosomal machineries through TFEB. The ISR has been reported to regulate autophagy at the transcriptional level through upregulating a number of autophagic genes.

1.1.3 The cellular functions and physiological relevance of autophagy

When initially discovered, autophagy was merely deemed as a bulk degradation process without much specificity. However, with increasing knowledge on the mechanism and function of autophagy, this simple notion is now being challenged and autophagy is being appreciated as a more selective process than initially thought. The both bulky and selective natures of autophagic degradation serve fundamental biological purposes, and its importance is demonstrated in its vast physiological and pathological implications, including neurodegenerative diseases, development, metabolic disorder, skeletal muscle function, cancer, and aging, *etc.* [127]. Generally, the functions of autophagy can be grouped into three facets: clearance of cellular waste, recycling of biomolecules and engagement in cell fate decision.

Basal autophagy, as well as induced autophagy under cytotoxic challenges, serves housekeeping functions by eliminating misfolded proteins, protein aggregates, and damaged organelles. Although somewhat misleading, this aspect of autophagic degradation is sometimes referred to as "selective autophagy", in which LC3, p62 and other autophagic molecules specifically direct substrates to the autophagosome for degradation [141]. A vast array of organelles, when damaged, undergo sequential encapsulation and degradation by autophagy via distinct mechanisms. Based on the organelle to be degraded, these specialized degradation processes are termed respectively as ERphagy [161], pexophagy [162], mitophagy [163] and lysophagy [164], *etc*. In particular, the ER is one organelle highly susceptible to the autophagic defect-caused disturbances such as accumulation of misfolded protein and aggregates. Autophagy maintains the homeostatic microenvironment of ER by performing protein quality control under normal conditions and protecting against ER catastrophe in case of overwhelming protein burden [161, 165-168]. Deleterious pathological conditions would ensue when the housekeeping role of autophagy is defective. In various tissues including the central nervous system, pancreas, skeletal muscle and liver, genetic deletion of *ATG* genes caused tissue damage and dysfunction, which shared common dyshomeostatic phenotypes such as the accumulation of protein aggregate and ER stress [169, 170]. In humans, a few prominent instances that are related to impaired autophagy are neurodegenerative diseases such as Alzhermer's disease and Parkinson's disease, in which the function of autophagy is abnormal and the consequent accumulation of macromolecule rubbish becomes a detrimental threat to the cell [127]. In a more common degenerative scenario, aging, the decline of the autophagic activity is a potential contributory factor that perpetrates the buildup of cytotoxic molecules and undermines self-renewal [171].

The second essential function of autophagy is recycling of cellular biomolecules. As the end point of autophagy, the lysosome contains a wide variety of digestive enzymes

including glycosidases, proteases, lipases and nucleases, and these hydrolases generate a diversity of products [172]. The autophagy-produced amino acids, carbohydrates, lipid, and nucleotides are channeled into various metabolic and cellular processes for macromolecule synthesis or energy production. At the tissue level, autophagy regulates energy storage and maintains metabolic balance, for instance, through the mobilization of lipid droplets in adipose tissue and the degradation of glycogen in the liver [126]. More importantly, autophagy is a self-sustaining means by which the whole body withstands short-term nutrient limitation. *In vivo*, upon the deprivation of nutrients, autophagosomes were observed in most tissues in young to adult mice at 24 or 48 hours [173]. In the liver of wide-type mice, autophagy generated free amino acids whose level peaked at 24 hours after starvation, and those amino acids were shunted to gluconeogenesis to sustain blood glucose level [174]. However, the surge in amino acids, especially branched chain amino acids (BCAAs), was not observed in liver-specific *ATG7* knockout mice, and this was associated with the decrease of blood glucose after long-term starvation [174]. Particularly, autophagy enables the neonate to survive the starvation period after birth and before breastfeeding. In wild-type neonatal mice, autophagy was activated within 30 minutes after birth, and it persisted at high levels for 3 to 12 hours, finally returning to basal level after 1 to 2 days of birth [175]. This temporally regulated autophagic flux is necessary for neonatal survival, since *ATG5* knockout mice, although born with a normal phenotype, died within one day after birth. These autophagy-deficient pups had tremendously low levels of amino acids in the plasma and in the heart, and their life could be prolonged by forced milk feeding [175]. Similar neonatal lethal phenotypes were also observed in *ATG*7-/- [176], *ATG3*-/- [177], *ATG16L1*-/- [178] and *ATG9*-/- [179] mice, which invariably died within 1 day of delivery without additional nutrient supply. Therefore, under adverse circumstances, autophagy is a critical source of nutrient that the cells and the whole body subsist on.

Apart from its role in recycling cellular constituents and mobilizing nutrient stores, autophagy makes life-or-death decisions under special circumstances. Autophagy is grouped as type II programmed cell death, as opposed to type I programmed cell death apoptosis. This term is nevertheless misleading, since autophagy can be either pro-survival or pro-death in different contexts [147]. Considering its role in removing cytotoxic components and providing nutrients, autophagy is pro-survival and promotes stress resistance under adverse conditions. However, autophagy can be an autonomous cell death pathway in certain circumstances [147], such as in the case of selective tissue clearance during development [180]. To further complicate this picture, the system of autophagy crosstalks with other cell death pathways including apoptosis and necrosis, and their relationship can be either cross-inhibitory or cross-activating [153, 181, 182]. The elusive effect of autophagy on cell fate is reflected in its paradoxical role in cancer: the activation of autophagy carries either pro-cancer functions or anti-cancer potentials [183]. Cellular and stress contexts are important aspects to consider when the biological consequence of autophagy is to be interpreted.

1.2 Skeletal muscle

1.2.1 Skeletal muscle as an amino acid storage organ

Skeletal muscle is a highly specialized tissue responsible for locomotion and maintaining posture in the body. Apart from this physical function, the high protein content and its lability qualify skeletal muscle as an amino acid reservoir in the body. The proteins in skeletal muscle constitute about 45% of total proteins in adult body, and the turnover rate of skeletal muscle proteins is estimated to be 12% [184]. The degradation of skeletal muscle protein is enhanced when there is a lack of nutrients, such as during the post-absorptive state and fasting [184]. The loss of skeletal muscle under long-term starvation constitutes a large proportion of total weight loss, and one salient example is the severe loss of muscle suffered by the victims during the famine period [184]. Autophagy releases amino acids from the skeletal muscle into the blood

stream and through circulation they feed into protein synthesis, gluconeogenesis and other metabolic processes in peripheral tissues [185]. In addition to the inter-organ nutrient supply, the recycling of amino acids also meets the demand for protein synthesis and energy production in skeletal muscle. Moreover, amino acids are not just substrates for metabolic processes but also messengers regulating signaling pathways, and therefore amino acids produced during proteolysis in skeletal muscle also reprogram a variety of cellular and physiological processes. As such, skeletal muscle is a maintainer of systematic amino acid balance and metabolic homeostasis.

1.2.2 Autophagy is a mobilizer of the amino acid pool in skeletal muscle

Degradation of skeletal muscle protein is carried out by two proteolytic pathways: the UPS and autophagy. In the UPS, targeted proteins are tagged with the polyubiquitin chain and sent to the 26S proteasome for degradation [186]. The UPS is a selective degradation process that targets misfolded proteins and controls protein turnover [186], although it also serves as an amino acid source [187, 188]. In skeletal muscle, while the UPS mainly degrades short-lived, soluble and myofibrillar proteins [189], autophagy targets long-lived proteins as well as protein aggregates and organelles [190]. Although both proteasomal degradation [191-193] and autophagy [173, 193, 194] can be activated in skeletal muscle under exercise and starvation, the latter is the principal avenue for this tissue to mobilize its internal nutrient store under nutrient restriction conditions. Autophagy is persistently activated in skeletal muscle during starvation, and *in vivo*, the GFP-LC3 transgenic mice showed differential patterns of autophagic flux in fast-twitch versus slow-twitch skeletal muscles under nutrient limitation [173]. After 24 hours of starvation, autophagy was preferentially induced in fast-twitch muscle, featured by the distribution of many small GFP-LC3 puncta between myofibrils and in the perinuclear regions, whereas few puncta were monitored in slow-twitch muscles. At 48 hours of starvation, the GFP-LC3 puncta persisted in fast-twitch muscles, and the slow-twitch muscles started to show mild signs of

autophagy, characterized by a small number of GFP-LC3 puncta in the periphery of myofibrils [173]. The generation of nutrients by autophagy in skeletal muscle under nutrient limitation is of great biological importance. In the fasting state, defect of autophagy in the skeletal muscle resulted in morphological abnormalities and shrinkage of muscle fibers [195], and it hampered the gluconeogenic process in the liver, resulting in hypoglycemia and hyperketosis [196]. Thus, autophagy underpins the regulatory role of skeletal muscle in systematic metabolism and enables timely and ample production of nutrients by this tissue.

1.2.3 Autophagy and skeletal muscle homeostasis

The basic unit of skeletal muscle is the sarcomere, in which myosin, actin and other proteins are aligned and sophisticatedly organized to form striated structures [197]. The contractile function of skeletal muscle and its regulatory effect on global metabolism rely on the precise synthesis and organization of proteins in this basic unit. Not only the quantity of muscle proteins but also the quality of any cellular component must be tightly controlled, and autophagy is a key executer of this "QC" process.

The dynamic balance of skeletal muscle protein is controlled by the dual regulation of protein synthesis and degradation. The mTORC1 pathway is a principal pathway contributing to protein synthesis and the maintenance of muscle mass. Genetic ablation of mTORC1 signaling through the deletion of mTOR [198], Raptor [199], or S6K1 [200] lead to a reduction in myofibril size and dystrophy. On the other hand, the rate and extent of muscle protein breakdown are regulated by both the UPS system and autophagy. While upregulated "atrogens" in the UPS system have been recognized as one causal factor for atrophy [201], aberrancy in the autophagic process is also a fundamental culprit of skeletal muscle wasting [202]. The latter scenario is not confined to the condition of excessively active autophagy, which exhausts the constitutive components of skeletal muscle and results in atrophy [203-206], but also occurs when autophagy cannot function normally. In humans, defects in lysosomal

degradation, which is the end stage of autophagy, cause myopathies as in the case of Danon disease and Pompe disease [207]. Muscle-specific *ATG* knockout mice displayed grave muscle loss and atrophy, which reinforced the indispensability of autophagy for maintaining skeletal muscle mass [195, 208]. Therefore, delicately controlled level of autophagy is the premise of muscle integrity and function.

Moreover, autophagy maintains the homeostatic microenvironment of skeletal muscle through the elimination of toxic cellular components and the renewal of cellular organelles. For instance, physical exercise is usually coupled with the production of reactive oxygen species production and the buildup of damaged proteins, aggregates and organelles [192, 209, 210], which are cytotoxic if not timely and efficiently eradicated. Autophagy is activated during exercise to provide energy for energy production and to degrade damaged cellular constitutes [211]. Autophagic defects lead to increased oxidative stress and the accumulation of damaged mitochondria during muscle contraction [212], and adversely affect performance after endurance exercise training [213]. In skeletal muscle-specific autophagy-defective mice, apart from the shared phenotype of muscle loss, their skeletal muscle also showed deleterious accumulation of protein aggregates and damaged organelles such as mitochondria and ER [195, 208]. In addition, the enhancement of basal autophagy which can accelerate the turnover of cellular components is an underlying benefit of regular exercise [211].

1.3 Rationale and hypotheses of this study

Autophagy performs essential housekeeping functions at the basal level and serves as a nutrient supplier under nutrient-limited conditions. mTORC1 [83-85] and the ISR [123, 148-152, 159] have been established as two regulators of autophagy. However, the impact of autophagy on these two pathways is less characterized. Autophagy produces biomolecules through degrading intracellular constituents in the absence of nutrient supply. I hypothesized that under nutrient limitation, autophagy was capable of producing sufficient nutrients to support mTORC1 signaling, and this phenomenon

might be mostly evident in a protein-abundant and metabolically flexible model such as skeletal muscle cells [184]. On the other hand, the dysfunction of autophagy might result in stress that activated the ISR system. Thus, I investigated the regulation of mTORC1 pathway and the ISR system by autophagy under amino acid starvation in mouse myotubes and determined the underlying mechanisms. This study would provide information on the interplay between mTORC1 and autophagy, and the critical role of autophagy in maintaining skeletal muscle homeostasis.

2. Materials and Methods

2.1 Materials

Chloroquine diphosphate and cycloheximide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bafilomycin A1, lactacystin and spautin-1 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Torin 2 was from Tocris Bioscience (Bristol, UK). Anti-MHC antibody was from Developmental Studies Hybridoma Bank (Iowa City, IA, USA). Antibodies specific for CHOP, LC3A, eIF2α, phospho-eIF2α (Ser51), S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), p70S6 kinase, phospho-p70S6 Kinase (Thr389), ubiquitin, anti-mouse IgG, HRP-linked antibody, and anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2 Cell Culture

Mouse C2C12 myoblasts were maintained at low confluency in growth medium, made from Dulbecco's modified Eagle's medium (DMEM, PAA Laboratories, GE Healthcare, Little Chalfont, Buckinghamshire, UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in the presence of 5% CO2. To induce differentiation, 70 - 80% confluent myoblasts were rinsed and incubated with differentiation medium consisting of DMEM supplemented with 2%

horse serum, 100 U/ml penicillin and 100 µg/ml streptomycin for 3 days. C2C12 myoblasts fully differentiated into myotubes on the third day of differentiation.

2.3 Cell Treatment

On the third day of differentiation, C2C12 myotubes were rinsed and treated in basal incubation media or glucose/amino acid deprivation media for indicated conditions. The basal incubation media consisted of Earle's balanced salts solution (EBSS) supplemented with 25mM glucose, 1 × MEM amino acid solution (Life Technologies, Carlsbad, CA, USA), 4 mM glutamine, 1 × MEM Vitamin Solution (Life Technologies, Carlsbad, CA, USA), 0.2% BSA, 0.22% weight/volume NaHCO3, 100 U/ml penicillin and 100 µg/ml streptomycin. Amino acid and glucose deprivation was conducted by the exclusion of 1 × MEM amino acid solution and 25 mM glucose, respectively. Autophagy was inhibited by treating the myotubes with 200 nM bafilomycin A1, 50 µM chloroquine, and 10 µM spautin-1 for indicated durations. Inhibition of the proteasome activity was performed by treating the myotubes with 8 µM lactacystin for 2 hours. To inhibit protein synthesis, myotubes were treated with 30 µM cycloheximide for 4 hours. Torin2, an ATP-competitive inhibitor of mTOR, was used at 0.25 µM for the indicated time to inhibit mTORC1 signaling.

2.4 Western Blot

After treatment under desired conditions in 6-well plates, myotubes were frozen using liquid nitrogen and stored at –80°C until processed. Frozen cells were lysed on ice with 200 µl RIPA buffer [25 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% vol/vol IGEPAL, 1 % wt/vol sodium deoxycholate, and 0.1% wt/vol SDS] supplemented with 5 mM β-glycerophosphate, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 5 mM sodium pyrophosphate and protease inhibitor cocktail (Pierce, Thermo Scientific, Waltham, MA, USA). Cell lysate was subjected to sonication (200 amplitude, 1sec per time for 3 times) to make the lysate less viscous. The lysate was then centrifuged at 12000 rpm at 4°C for 10 min, and supernatant was transferred to a new centrifuge tube to remove

cell debris. The total protein content of the homogenate was determined using the Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA). Proteins were diluted to 1µg/µl with Milli Q water, denatured in sample buffer [32.5 mM Tris·HCl (pH 6.8), 2.5% vol/vol glycerol, 1% wt/vol SDS, 0.005% wt/vol bromophenol blue, and 50mM dithiothreitol] and heated for 5 min at 65°C. 30 µg protein was loaded into each lane of 10% SDS-PAGE gel, and gels were run at 100V until the dye front disappeared. Proteins were transferred from the gel to Immun-Blot PVDF membrane (BioRad, Hercules, CA, USA) using the wet transfer method (115V at 4°C for 1 hour). Membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) at room temperature for 1 h, and then incubated with respective primary antibodies (diluted in TBST with 1%BSA) overnight at 4°C. The next day, after being rinsed with TBST for 30 min, membrane was incubated with HRP-conjugated secondary antibody for 1 hour at room temperature, followed by rinse with TBST for 30min. The membrane was incubated with ECL (Pierce, Themo Scientific, Waltham, MA, USA) to generate the chemiluminescence signal. Band intensities were quantified using image analysis software. For figures with both longer and shorter exposure bands, "longer exposure" (long exp) was utilized for quantification.

2.5 Amino Acid Analysis.

For measuring intracellular level of amino acids, myotubes were treated on 100-mm plate under indicated conditions. Myotubes were homogenized in 450 µl of 10% 5-sulfosalicylic acid (Sigma-Aldrich, St. Louis, MO, USA) on ice. Cell lysate was transferred to centrifuge tube, and then subjected to sonication (200 amplitude, 1sec per time for 3 times), followed by centrifugation at 12000 rpm at 4°C to remove cell debris. The supernatant was collected and amino acid concentrations were analyzed by HPLC at Vanderbilt University Hormone Assay & Analytical Services Core (Nashville, TN, USA). The pellet was collected, dissolved in 500 µl RIPA buffer [25

mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% vol/vol IGEPAL, 1 % wt/vol sodium deoxycholate, 0.1% wt/vol SDS] supplemented with 5 mM β-glycerophosphate, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 5 mM sodium pyrophosphate and protease inhibitor cocktail and subjected to sonication. Protein concentration was measured using the Pierce BCA protein assay kit. The concentration of each amino acid was normalized to the total protein content, and the amino acid level was expressed as μmol/ μg protein for bar graph and nmol/ μg protein for table.

2.6 Quantitative Real-time PCR.

For qPCR analysis of gene transcription, C2C12 myotubes were treated in 6-well plates with or without bafilomycin A1 (200 nM) in the presence or absence of amino acids. RNA was purified using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's recommendations, and 1 μg total RNA was subjected to reverse transcription using the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). A reaction mix containing specific primers for target genes, SYBR Green Select Master Mix (Applied Biosystems, Carlsbad, CA, USA), and cDNA template was prepared, and real-time PCR was performed on an ABI 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The PCR program is: one cycle of 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1min. After these reactions, the specificity of amplification was assessed using dissociation curves. The primer sequences for genes analyzed in this study are listed in Table 1. For data analysis, the relative quantitative method was used, by normalizing the mRNA level of target genes against that of the housekeeping gene, β-actin.

2.7 Monitoring GFP-LC3 Puncta by Confocal Microscopy

Sparsely growing mouse C2C12 myoblasts were transfected with GFP-LC3 expression vector (Cell Biolabs, San Diego, CA, USA) using Lipofectamine 2000 (Invitrogen, life technologies) and selected with 750 μg/ml G418 (Life Technologies). Selected myoblasts were transferred to a 35 mm imaging dish (Ibidi, Martinsried, Germany) at

a subconfluent density and induced to differentiation. Amino acid starvation was performed in amino acid deprivation medium, and GFP-LC3 puncta was examined in live myotubes (maintained in a 37°C chamber with 5% CO2) under a Zeiss LSM 710 confocal microscope (Jena, Germany) with a ×40 oil-immersion objective. To monitor the change in GFP-LC3 puncta formation, images were captured at various time points after amino acid deprivation.

2.8 Statistical Analysis

Data are expressed as the mean ± S.E.M of 6 independent replicates. Differences among groups were determined by ANOVA followed by Fisher's least significant differences *post hoc* analysis. Statistical significance was accepted at $p < 0.05$.

Table 1. Primer sequences for real-time PCR in the first study

Target Gene	Description	Primer Sequence		Genebank Accession No.
		FWD (5' to 3')	REV (5' to 3')	
ACTB	Mus musculus actin, beta	CCAACCGTGAAAAGATGAC	CAGCCTGGATGGCTACGTACA	Mouse NM_007393
GPT (Alt)	Mus musculus glutamic pyruvic transaminase, soluble	ACTTCCATGCTAAATTCA	AATCAGAAATAGTGAGGG	Mouse NM_182805
GOT1 (Ast)	Mus musculus glutamate oxaloacetate transaminase 1, soluble	GGACTTGGTCTCACATCA	AGATAGATATGCTTCTCGTTGA	Mouse NM_010324
GLUD1	Mus musculus glutamate dehydrogenase 1	GGCCTACACAATGGAGAG	GATAGCATTGACATAGGCAG	Mouse NM_008133
SLC7A1	Mus musculus solute carrier family 7 (cationic amino acid transporter, y+ system),	AGGCATCATCTGGAGACA	ATGAGATAGATGTTCACGAAGA	Mouse NM_007513
TRIB3	Mus musculus Tribbles homolog 3	GCGTCGCTTTGTCTTCAG	TTGTCCCACAGAGAGTCAT	Mouse NM_175093

3. Results

3.1 Inhibition of autophagy suppresses mTORC1 signaling under amino acid starvation and induces the ISR

It is well-known that mTORC1 is an upstream regulator of autophagy. Under starvation conditions, the inhibition of mTORC1 releases several initiators of autophagy, such as ULK1/2 and ATG13 [83-85]. On the other hand, as another nutrient- and stress-sensitive signaling pathway, the ISR has also been recognized as a regulator of the autophagic system [123, 148-152, 159]. However, it is less clear whether the activity of autophagy, as a nutrient-generating and housekeeping machinery, has an impact on the two nutrient-sensitive signaling networks. To investigate the effect of autophagy on these two pathways, C2C12 myotubes were treated in basal or amino acid deprived conditions, and the activity of autophagy was inhibited using the lysosomal v-ATPase inhibitor bafilomycin A1 (Baf), which prevents the fusion between autophagosome and lysosome [214]. LC3 was used as a marker for the autophagy flux [215]. Since the conversion of LC3AI to LC3AII is a dynamic process, the LC3AII: LC3AI ratio must be assessed in the absence and presence of end-stage autophagy inhibitors to validate the increased formation of autophagosomes [215]. Thus, in our experimental settings, the usage of end-stage autophagy inhibitors served dual purposes including both the inhibition of autophagic degradation and the confirmation of LC3AII accumulation. Baf strikingly inhibited the degradation of LC3AII (Fig 6A), suggesting its efficacy in inhibiting autophagic degradation. Amino acid deprivation for 2 hours increased the amount of LC3AII under normal conditions, which was further accumulated in the presence of Baf (Fig 6A), indicating the activation of autophagy under amino acid starvation. In contrast to amino acid starvation, glucose deprivation for 2 hours neither increased LC3AII accumulation on its own, nor did it elevate autophagic flux during amino acid restriction (Fig 6A). This suggested that the autophagic system in mouse myotubes is more sensitive to amino acid limitation as compared to glucose deficiency.

At 4 hour of amino acid starvation, though, the accumulation of LC3AII under amino acid deprivation was not significant both in the presence and absence of Baf treatment (Fig 6B, the explanation is provided in the next section). mTORC1 signaling activity relies on the presence of adequate amino acids in the cell [216]. Interestingly, after 4 hours of amino acid starvation, there was no significant decline in mTORC1 signaling activity, demonstrated by the mild reduction in the phosphorylation of its downstream effectors p70S6K (T389) (Fig 6C) and S6 (S235/236) (Fig 6D). However, mTORC1 signaling activity was dramatically decreased when autophagy was inhibited under this condition, featured by the reduction of phospho-p70S6K (T389) (Fig 6C) and phospho-S6 (S235/236) (Fig 6D). It was notable that autophagy inhibition alone did not have an obvious effect on mTORC1 signaling under amino acid-sufficient conditions (Fig 6C and 6D). These results suggested that the activity of autophagy is required for sustaining mTORC1 signaling activity under amino acid limitation. Unlike its effect on mTORC1 signaling, inhibition of autophagy by Baf induced the phosphorylation of eIF2α (S51), which is the hallmark of the ISR, regardless of extracellular amino acid availability (Fig 6E), indicating that autophagy failure itself represents as a drastic stress that elicits this stress response. Nonetheless, amino acid deprivation did not activate the ISR (Fig 6E), suggesting that mouse myotubes have the intrinsic ability to maintain homeostasis under amino acid restriction. Taken together, autophagy maintains mTORC1 signaling under amino acid limitation, whereas it negatively regulates the ISR independent of amino acid availability.

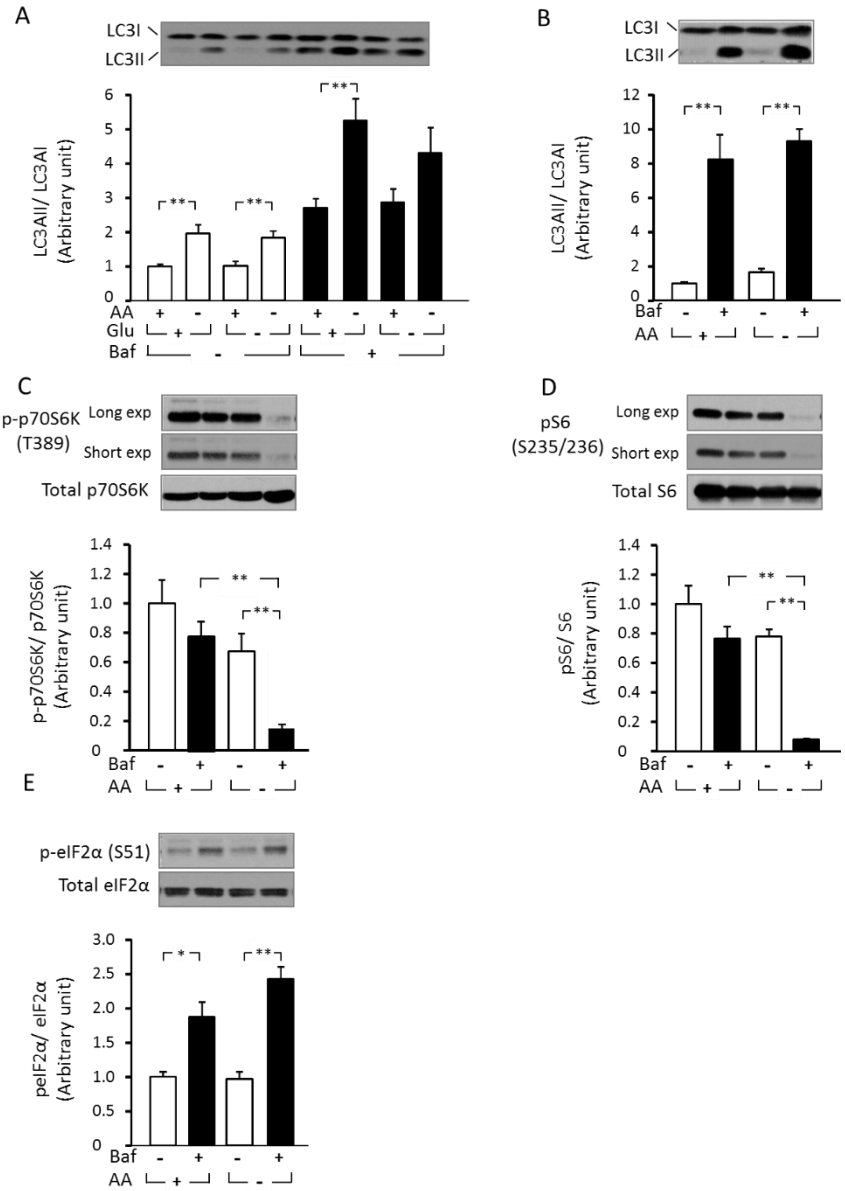


Fig 6. The effects of autophagy inhibition on the mTORC1 signaling and the ISR. (A) C2C12 myotubes were treated in basal condition or in amino acid (AA)- or glucose (Glu)-deprived conditions for 2 hours. Baf was added at the final 1 hour. Immunoblotting was applied to examine the level of LC3AII/LC3AI. (B, C, and D) C2C12 myotubes were treated with or without Baf in the presence or absence of amino acids (AA) for 4 hours. Immunoblotting was performed to evaluate the level of (B) LC3AII/LC3AI, (C) phospho-p70S6K (T389)/p70S6K, (D) phospho-S6 (S235/236)/S6, and (E) phospho-eIF2α (S51)/eIF2α. Data are expressed as fold of control. Long exp, longer exposure; Short exp, shorter exposure. Data are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.2 Dynamics of mTORC1 signaling and autophagic flux during amino acid deprivation

The previous results showed that mTORC1 signaling activity was sustained by autophagy at 4 hour of amino acid starvation (Fig 6C and 6D). I next monitored the dynamics of the mTORC1 and ISR signaling as well as autophagic flux throughout the 4 hours of amino acid deprivation. mTORC1 signaling displayed a biphasic pattern under amino acid starvation: phospho-p70S6K (T389) was first suppressed at 0.5 and 1 hour, and then gradually recovered from 2 to 4 hours, resuming its basal activity at 4 hour (Fig 7A). Consistent with mTORC1 signaling activity, the activity of autophagy, as shown by the LC3AII: LC3AI ratio, first increased after amino acid deprivation from 0 to 1 hour, and then progressively declined, reaching a minimum level by 4 hour of starvation (Fig 7A). This result is consistent with our previous LC3 data at 4 hour of amino acid starvation (Fig 6B). To further evaluate the dynamic flux of autophagy, I monitored the formation of GFP-LC3 puncta by fluorescence microscopy. Consistent with western blot result, GFP-LC3 puncta increased from 0 to 1 hour of amino acid starvation, and then decreased from 2 to 4 hours (Fig 7B). When autophagy was inhibited using Baf, though, the activity of mTORC1 signaling failed to be recovered during 2 to 4 hours of amino acid starvation, and the autophagy flux constantly increased from 0 to 2 hours and persisted afterwards (Fig 7A). With respect to the ISR, phosphorylation of eIF2α (S51) was not altered by amino acid deprivation, which was consistent with the previous data (Fig 6E). However, the ISR was rapidly and persistently induced by autophagy inhibition, which began as early as 1 hour (Fig 7A), demonstrating that the ISR system is sensitive to the stress induced by autophagic defect. The temporally inverse relationship between mTORC1 signaling and autophagic flux and the dependence of mTORC1 signaling recovery on autophagy implied that autophagy may produce amino acids which become sufficient to reactivate mTORC1 at the later time points of amino acid limitation.

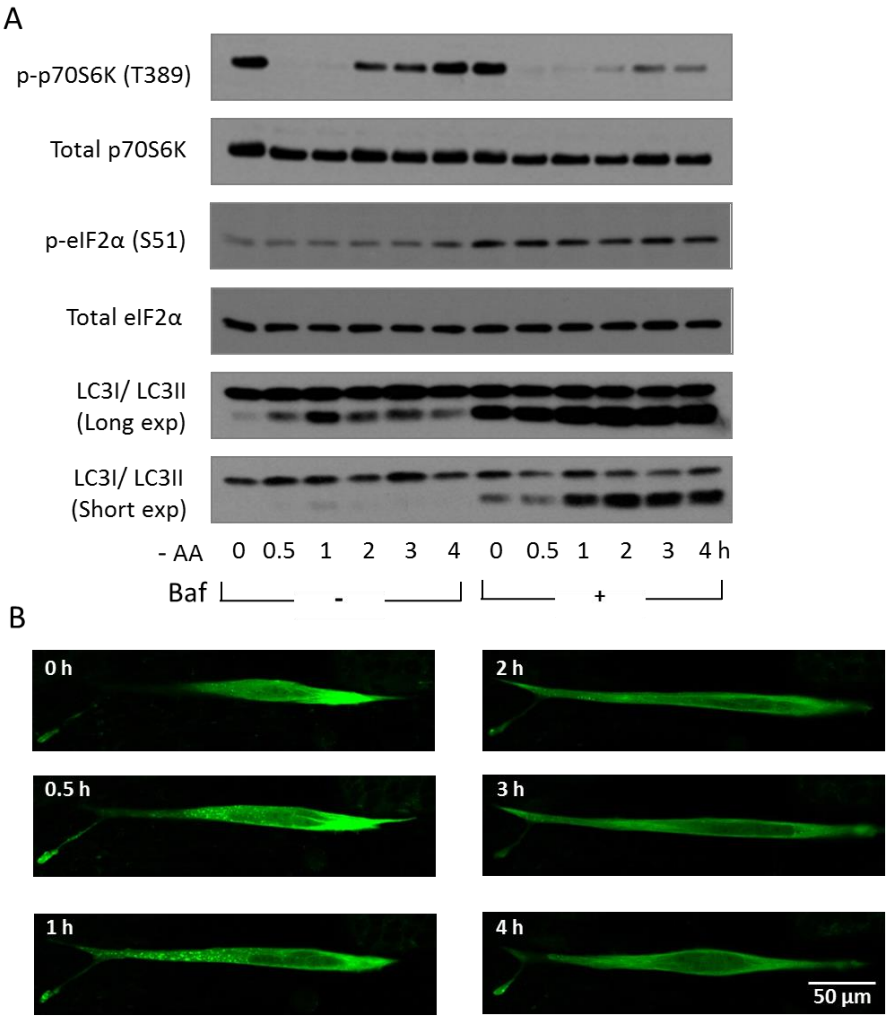


Fig 7. The dynamics of mTORC1 activity, eIF2α phosphorylation and autophagic flux after amino acid deprivation. (A) C2C12 myotubes were deprived of amino acids for various durations in the presence or absence of Baf. Immunoblotting was used to examine the level of indicated proteins and their phosphorylation. (B) C2C12 myotubes which stably express GFP-LC3 were deprived of amino acids for various durations, and GFP-LC3 puncta was monitored under fluorescence microscope. Long exp, longer exposure; Short exp, shorter exposure.

3.3 Amino acid supplementation rescues mTORC1 signaling but not the ISR under autophagy inhibition

As mentioned above, Baf inhibits the end stage of autophagy via targeting v-ATPase at the lysosome [214]. There is report that the activation of mTORC1 by amino acids depended on the activity of v-ATPase [18]. To exclude the possibility that the inactivation of mTORC1 by Baf under amino acid limitation was due to the defect of v-ATPase, I used an alternative inhibitor of autophagy chloroquine (CQ), which blocks

the progression of autophagy at the late stage by elevating intralysosomal pH [217]. Our previous data suggested that autophagy might activate mTORC1 through releasing amino acids (Fig 7A). To test this hypothesis, I deprived myotubes of amino acids for 3 hours and then re-supplied with or without amino acids for 1 hour in the presence or absence of CQ. CQ effectively inhibited autophagic degradation, as evidenced by the accumulation of LC3AII (Fig 8D). The phosphorylation of p70S6K (T389) and S6 (S235/236) was preserved under amino acid deprivation, but dramatically declined when CQ was administered (Fig 8A and 8B), in agreement with the phenotype under Baf treatment (Fig 6C and 6D). The inhibition of mTORC1 signaling by CQ in the absence of amino acids was rescued by 1 hour of amino acid re-addition (Fig 8A and 8B), which suggested that the decline in intracellular amino acids was responsible for the inactivation of mTORC1 under autophagy inhibition. However, the activation of eIF2α by CQ was not relieved by amino acid supply (Fig 8C), indicating that the induction of the ISR during autophagy blockage is not due to amino acid inadequacy.

Both Baf and CQ inhibit autophagy at late stages [214, 217]. Then I tested whether the inhibition of autophagy at the early stage also affected mTORC1 signaling under amino acid restriction. Spautin-1 inhibits the formation of autophagosome through impairing the Vps34 complex integrity [218]. In C2C12 myotubes, spautin-1 hindered the induction of autophagy under both basal and amino acid-restricted conditions (Fig 8G). Consistently, spautin-1 significantly suppressed phospho-p70S6K (T389) under amino acid deprivation (Fig 8E), confirming the requirement of autophagy for mTORC1 signaling activity during amino acid limitation. Nevertheless, phospho-eIF2α (S51) was not increased by spautin-1 (Fig 8F), which might be prevented by the activity of microautophagy and CMA which are insensitive to spautin-1. Collectively, these data further supported the notion that autophagy supports mTORC1 signaling under amino acid limitation through its capacity to release amino acids.

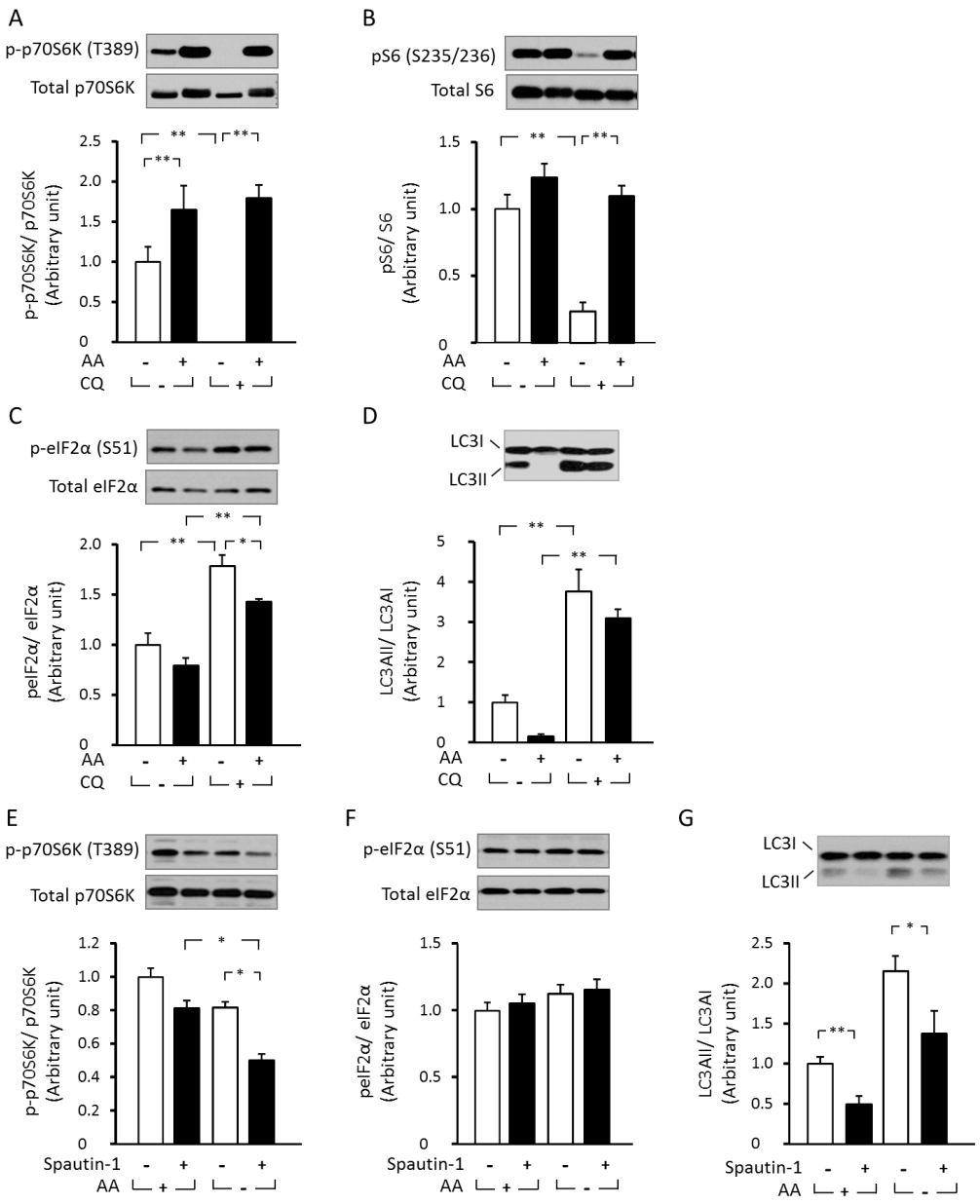


Fig 8. Amino acid supplementation restores mTORC1 activity under autophagy blockage. (A, B, C and D) C2C12 myotubes were treated with or without CQ in amino acid-deprived conditions for 3 hours, and further treated for 1 hour with or without amino acid re-addition. Immunoblotting was performed to assess (A) phospho-p70S6K (T389)/p70S6K, (B) phospho-S6 (S235/236)/S6, (C) phospho-eIF2α (S51)/eIF2α, and (D) LC3AII/LC3AI. (E, F and G) C2C12 myotubes were pre-treated with or without spautin-1, and treated with or without spautin-1 in the presence or absence of amino acids (AA) for 4 hours. Immunoblotting was applied to examine (E) phospho-p70S6K (T389)/p70S6K, (F) phospho-eIF2α (S51)/eIF2α, and (G) LC3AII/LC3AI. Data are expressed as fold of control, and are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.4 Inhibition of protein synthesis rescues mTORC1 signaling during autophagy blockage

Intracellular amino acid balance is regulated by amino acid expenditure by biosynthetic processes such as protein synthesis, and the input into the intracellular amino acid pool from extracellular uptake and intracellular degradation processes. Our previous data supported that autophagy maintains mTORC1 signaling probably by contributing to the intracellular amino acid pool under amino acid starvation. To further evaluate this notion, I inhibited protein synthesis as an approach to elevate intracellular amino acids, and examined whether it could rescue amino acid signaling pathways when autophagy was blocked during amino acid deprivation. I used cycloheximide (Chx) to inhibit protein synthesis, which impedes tRNA translocation during the elongation phase [219]. Indeed, while the blockage of autophagy by Baf markedly suppressed the phosphorylation of p70S6K (T389) and S6 (S235/236) under amino acid starvation, their phosphorylation under this condition was potently rescued by Chx (Fig 9A and 9B). In parallel, the elevation of intracellular amino acid level by Chx decreased the autophagic flux under amino acid limitation (Fig 9D). Chx also evidently decreased the phosphorylation of eIF2α (S51) under both autophagy-sufficient and –deficient conditions (Fig 9C), indicating that the reduction in protein translation could alleviate the ISR. Thus, these data suggested that the repletion of intracellular amino acid pool by autophagy accounts for the activation of mTORC1 under amino acid-restricted conditions.

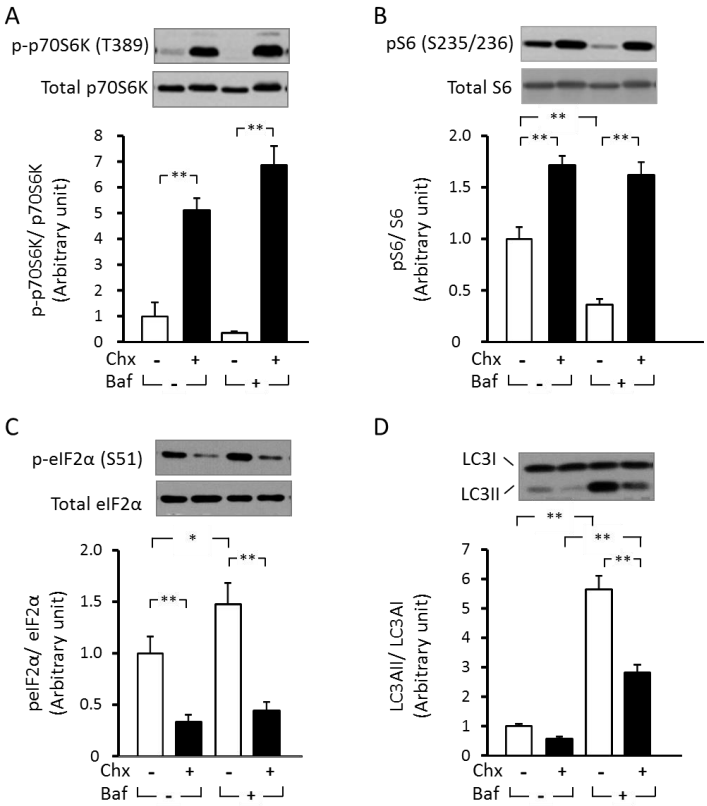


Fig 9. Inhibition of protein synthesis rescues mTORC1 activity and relieves the ISR under autophagy inhibition in the absence of amino acid supply. C2C12 myotubes were treated with or without Chx in the presence or absence of Baf for 4 hours under amino acid limitation. Immunoblotting was performed to evaluate (A) phospho-p70S6K (T389)/p70S6K, (B) phospho-S6 (S235/236)/S6, (C) phospho-eIF2α (S51)/eIF2α, and (D) LC3AII/LC3AI. Data are expressed as fold of control, and are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.5 Autophagy predominates over proteasome in the regulation of amino acid balance in mouse myotubes

The UPS [187, 188] and autophagy [174, 175] are two principal proteolytic systems which degrade intracellular components and generate amino acids under nutrient-limited conditions. While the UPS degrades short-lived proteins [220], autophagy breaks down long-lived proteins as well as bulky aggregates and organelles [190]. Our previous data illustrated a critical role of autophagy in providing amino acids and sustaining mTORC1 activity under amino acid deprivation in C2C12 myotubes. Here I sought to evaluate the relative contribution of the UPS and autophagy to the intracellular amino acid pool by examining their effects on mTORC1 signaling. Lactacystin (Lac) inhibits the proteolytic activity of proteasome through binding to its catalytic subunits [221]. Treatment with Lac for 2 hours effectively blocked the degradation of ubiquitin-conjugated proteins in C2C12 myotubes, while the administration of Baf did not have any appreciable effect on the degradation of ubiquitinated proteins (Fig 10A). Likewise, the inhibition of proteasome by Lac did not interfere with the activity of autophagy (Fig 10C). After 2 hours of amino acid deprivation, inhibition of autophagy led to a suppression of mTORC1 signaling marked by the reduction of phospho-p70S6K (T389) (Fig 10B), although to a lesser extent than that under 4 hours of amino acid limitation (Fig 6C). In contrast, the blockage of proteasomal degradation had no discernable impact on mTORC1 pathway (Fig 10B). Under amino acid starvation, neither did Lac impose any effect on phospho-p70S6K (T389) on its own, nor did it have any additional effect on phospho-p70S6K (T389) in combination with Baf (Fig 10B). Thus, autophagy serves as the predominant source of amino acids and the major contributor to the mTORC1 signaling in myotubes under nutrient-restricted conditions.

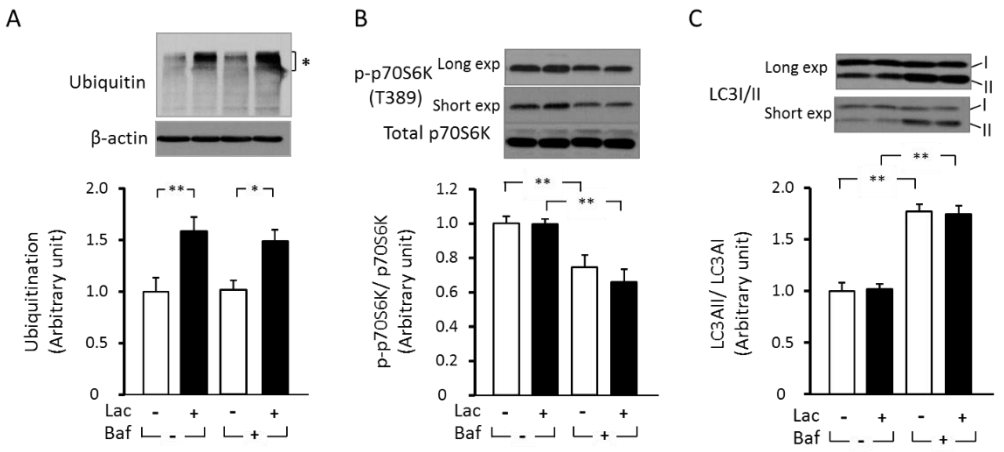


Fig 10. Proteasome inhibition has minimal effect on mTORC1 signaling under amino acid limitation. C2C12 myotubes were treated with or without Baf in the presence or absence of Lac for 2 hours under amino acid deprivation. Immunoblotting was used to evaluate (A) ubiquitinated proteins (asterisk highlights their accumulation), (B) phospho-p70S6K (T389)/p70S6K, and (C) LC3AII/LC3AI. Data are expressed as fold of control. Long exp, longer exposure; Short exp, shorter exposure. Data are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.6 Autophagy maintains amino acid homeostasis

Our data provided evidence that autophagy preserves the amino acid-dependent mTORC1 signaling during amino acid limitation. To assess the exact contribution of autophagy to amino acid balance, I measured the concentration of individual amino acids in C2C12 myotubes under autophagy-proficient or –deficient conditions in the presence or absence of amino acid supply. The amino acid analytical technique (by HPLC) that was used in this study could detect all the proteinogenic amino acids except for tryptophan and cysteine. The results demonstrated differential response patterns for non-essential amino acids (NEAAs) and essential amino acids (EAAs) in these conditions (Fig 11). Notably, treatment with Baf did not affect the level of any single amino acid in the presence of amino acid supply (Fig 11 and Table 2), suggesting that autophagy has little effect on amino acid balance under basal conditions. Under amino acid deprivation, all NEAAs increased dramatically above the normal levels, and the paradoxical surge was strikingly abrogated by the autophagy inhibitor Baf (Fig 11A). These results suggested that autophagy generated relatively excess NEAAs to meet the demand of protein synthesis and metabolic processes during amino acid limitation.

With regards to EAAs, the level of threonine mildly decreased under amino acid starvation, which was further reduced significantly by Baf (Fig 11B). Similarly, the level of lysine, although maintained during amino acid withdrawal, diminished tremendously under Baf treatment during amino acid restriction (Fig 11B). The signatures of threonine and lysine demonstrated that autophagy is vital for the preservation of their cellular content in the absence of amino acid supply. The level of the other eight EAAs fell below the detection limit under amino acid starvation, and remained undetectable under Baf treatment (Table 2). In spite of the decline in the eight EAAs at 4 hour of amino acid starvation, the preservation of threonine and lysine and the marked increase in all NEAAs highlighted the critical function of autophagy in maintaining amino acid homeostasis under nutrient restriction.

Table 2. Average concentration of individual essential amino acids in myotubes

(nmol/μg protein)

Amino acid	+AA		-AA	
	Veh	Baf	Veh	Baf
Arginine	11.0 ± 2.0	12.7 ± 1.4	ND	ND
Histidine	5.7 ± 1.5	6.1 ± 0.5	ND	ND
Leucine	6.7 ± 3.0	7.8 ± 2.3	ND	ND
Isoleucine	13.2 ± 3.4	14.2 ± 1.3	ND	ND
Methionine	4.0 ± 0.9	4.2 ± 0.4	ND	ND
Phenylalanine	8.0 ± 1.9	8.9 ± 1.0	ND	ND
Tyrosine	11.8 ± 3.4	11.3 ± 1.4	ND	ND
Valine	14.4 ± 3.5	15.0 ± 1.7	ND	ND

Cultured C2C12 myotubes were treated with vehicle (Veh) or bafilomycin A1 (Baf) in the presence or absence of amino acids for 4 h, and intracellular amino acid concentration was determined by HPLC. Data are means ± S.E.M. for n = 6 samples. ND, not detectable.

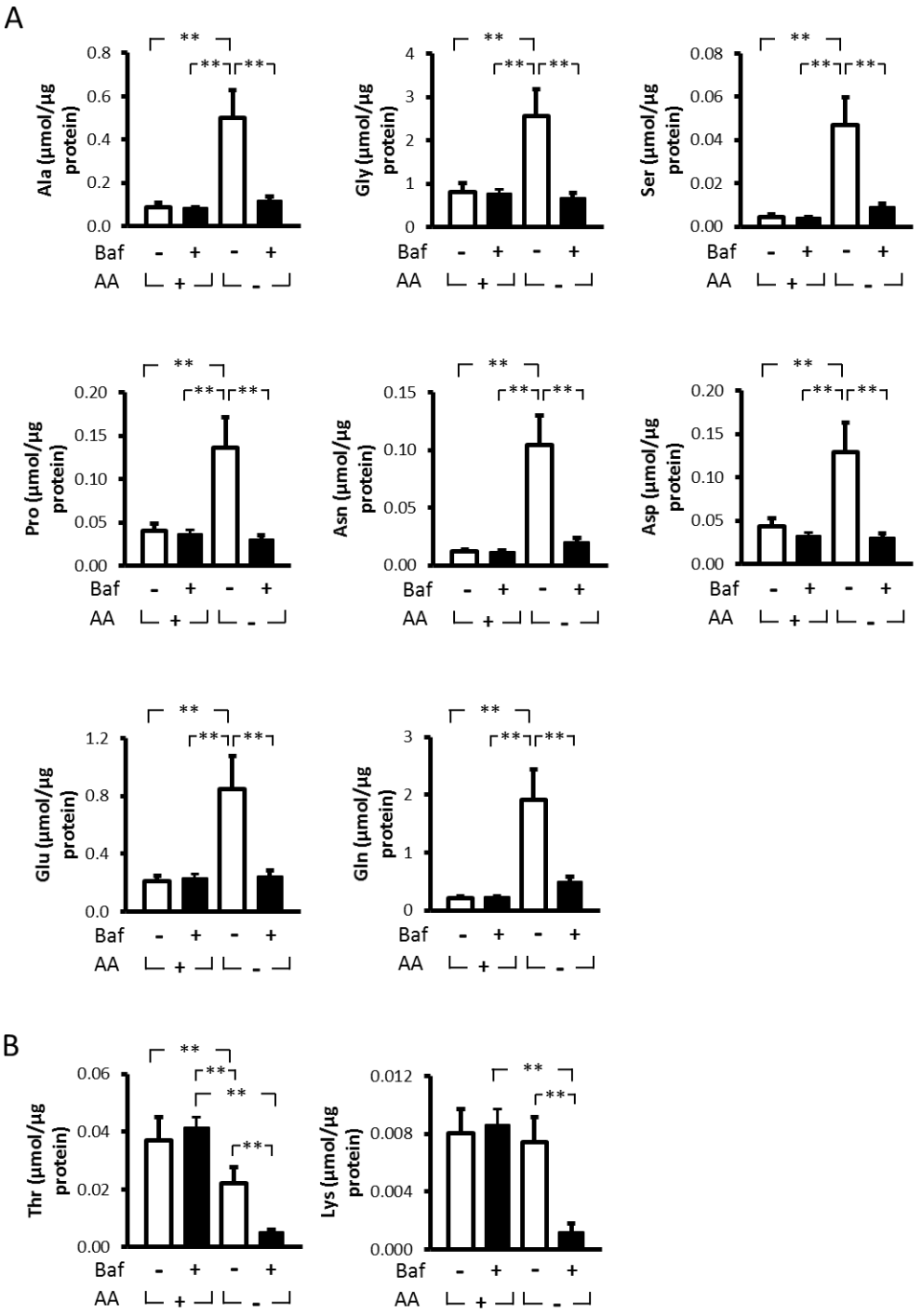


Fig 11. Autophagy maintains intracellular amino acid balance during amino acid limitation. C2C12 myotubes were treated with or without Baf in the presence or absence of amino acids (AA) for 4 hours. Intracellular amino acid concentration was determined by HPLC. (A) Ala, alanine; Gly, glycine; Ser, serine; Pro, proline; Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Gln, glutamine; (B) Thr, threonine; Lys, lysine. Data are expressed as micromoles per micrograms of protein, and are the mean±SEM for n = 6 samples. **P< 0.01.

3.7 Autophagy prevents the ISR

GCN2 and PERK are two upstream kinases in the ISR pathway, which are specific sensors for amino acid limitation and ER stress, respectively [94]. During nutrient scarcity, amino acid deficiency activates GCN2, while the lack of glucose can induce ER stress due to the inhibition of protein post-modification processes [222]. Nonetheless, C2C12 myotubes managed to avoid the ISR in the absence of amino acid supply (Fig 6E and 7A). Herein I sought to examine whether glucose starvation would activate the ISR in C2C12 myotubes and studied the role of autophagy in this setting. Glucose deprivation activated the ISR, evidenced by the increase in phospho-eIF2α (S51) and CHOP (Fig 12A and 12B). Of note, autophagy inhibition induced the ISR in nutrient-rich conditions and significantly exacerbated this stress response under glucose deprivation (Fig 12A). Consistent with our previous data at 2 hour of glucose starvation (Fig 6A), glucose deprivation for 4 hours did not mobilize autophagy (Fig 12C). It is likely that basal autophagy attenuates the ISR and prevents stress.

The data obtained so far suggested a protective role of autophagy against the ISR (Fig 6E, 7A, 12A and 12B). I then attempted to determine whether the enhancement of the autophagic process was able to reduce the basal ISR signaling. To augment the activity of autophagy, I inhibited its upstream regulator mTORC1 with Torin2. Torin2 abolished the phosphorylation of p70S6K (T389) (Fig 12F), confirming the effective inhibition of mTORC1 activity. Autophagy was activated by Torin2, as indicated by the dynamic conversion of LC3AI to LC3AII (Fig 12E). The enhancement of autophagy progressively reduced eIF2α (S51) phosphorylation (Fig 12D), suggesting that autophagy counterbalances this stress response system. Thus, autophagy is a stress-prevention system which can alleviate the ISR.

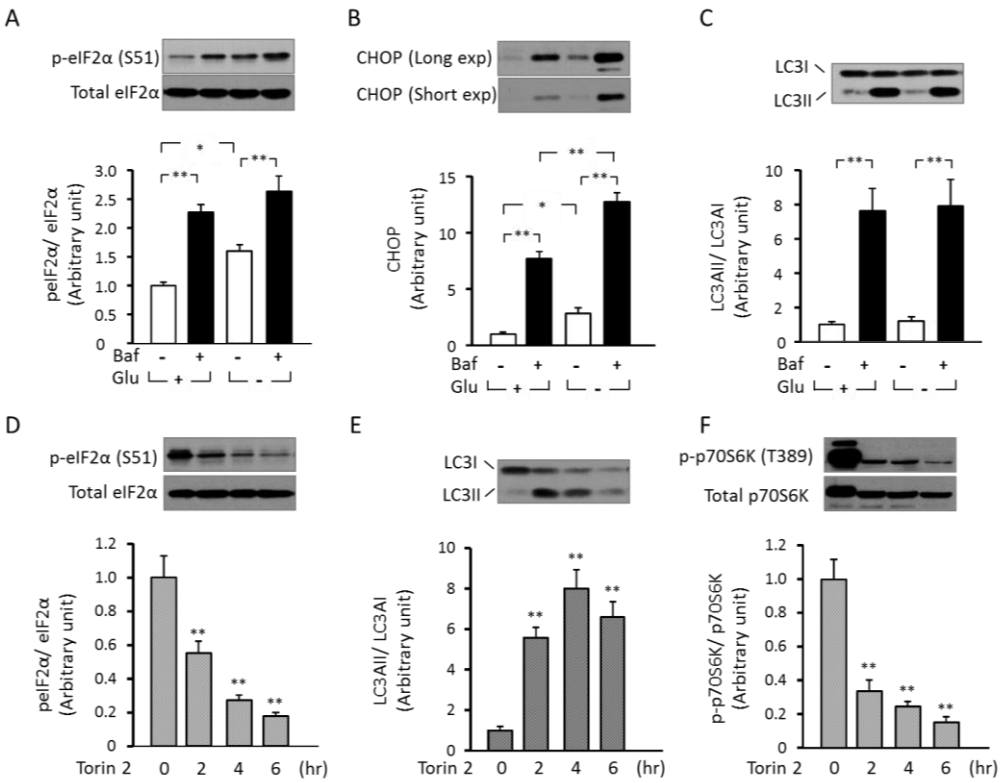


Fig 12. Autophagy prevents the induction of the ISR. (A, B and C) C2C12 myotubes were treated with or without Baf in the presence or absence of glucose (Glu) for 4 hours. Immunoblotting was performed to evaluate (A) phospho-eIF2α (S51)/eIF2α, (B) CHOP, and (C) LC3AII/LC3AI. (D, E, and F) C2C12 myotubes were treated with Torin 2 for indicated durations. Immunoblotting was performed to evaluate (D) phospho-eIF2α (S51)/eIF2α, (E) LC3AII/LC3AI, and (F) phospho-p70S6K (T389)/p70S6K. Data are expressed as fold of control at 0 hour. Long exp, longer exposure; Short exp, shorter exposure. Data are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.8 Autophagy inhibition alters the transcription of genes in amino acid metabolism

The perturbation in amino acid pool would trigger an alteration in amino acid metabolism [223, 224], which is one of the major processes targeted by the ISR system under stress [101, 223]. Given that autophagy is closely linked to amino acid balance and the stress signaling of the ISR, I evaluated the transcription of amino acid metabolic genes. In line with the signaling activity of the ISR (Fig 6E), the transcription of the genes in amino acid metabolism did not change during amino acid starvation (Fig 13A and 13B). In contrast, inhibition of autophagy by Baf significantly upregulated *TRIB3* and *SLC7A1* (Fig 13A), which are downstream targets of the ISR system, along with

GPT (alanine aminotransferase, Alt), *GOT1* (aspartate aminotransferase, Ast), and *GLUD1* (Fig 13B), which are key amino acid metabolic genes regulating the transfer of amino group. All these genes were induced by autophagy inhibition independent of amino acid availability (Fig 13). Therefore, while mouse myotubes maintain a normal amino acid metabolic profile under amino acid limitation, they are still sensitive to autophagy dysfunction and consequently reprogram the transcription of amino acid metabolic genes.

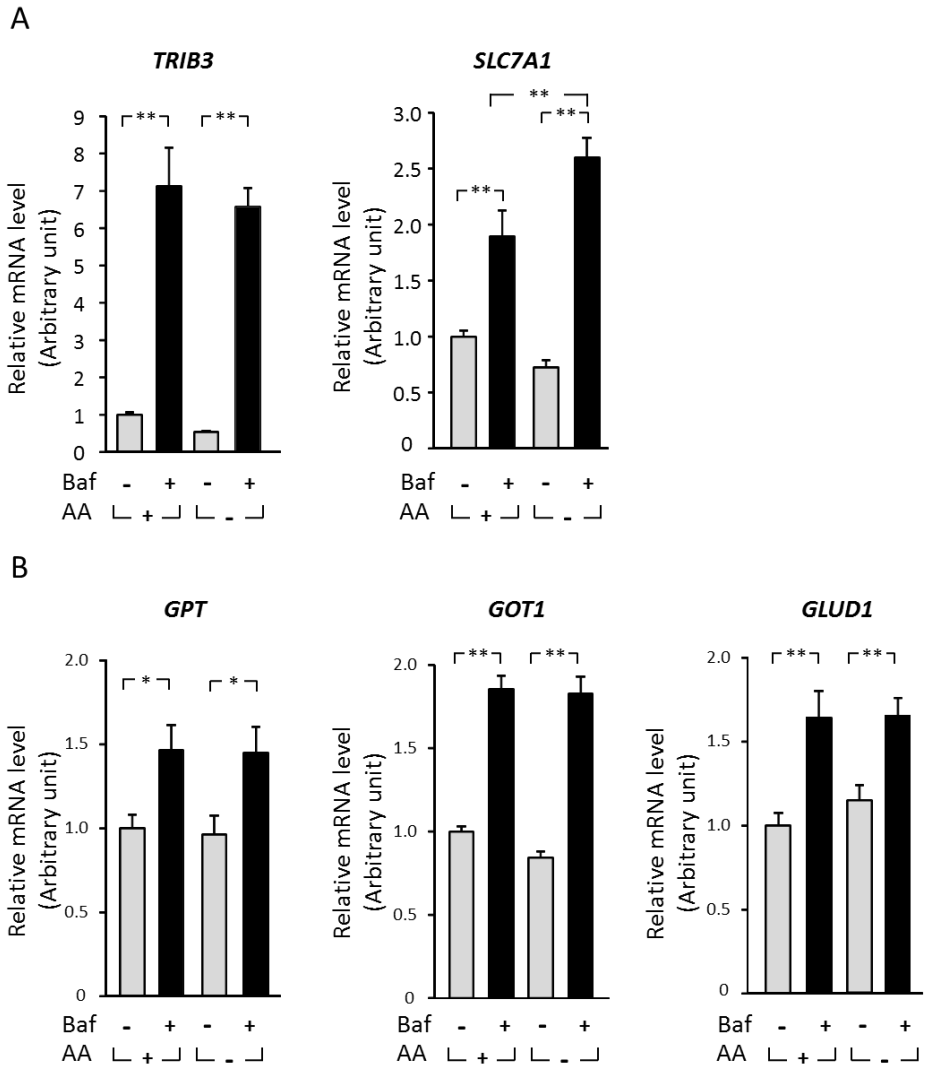


Fig 13. Autophagy regulates the transcription of amino acid metabolic genes. C2C12 myotubes were treated with or without Baf in the presence or absence of amino acids (AA) for 4 hours. The abundance of mRNA was analyzed by real-time PCR. The mRNA level of target genes was normalized against that of β-actin, and data are expressed as fold of control. Data are the mean±SEM for n = 6 samples. *P<0.05; **P< 0.01.

3.9 Chronic inhibition of autophagy impairs mTORC1 signaling and induces stress in mouse myotubes

The function of autophagy determines skeletal muscle homeostasis, and *in vivo*, autophagic defect resulted in pronounced loss of skeletal muscle mass [195]. Acute inhibition of autophagy (for 4 hours) suppressed mTORC1 signaling only in amino acid-deprived but not in normal nutritional conditions (Fig 6C and 6D). I next asked whether long-term blockage of autophagy would disrupt mTORC1 signaling under normal nutritional conditions. The accumulation of LC3AII verified the inhibition of autophagic degradation (Fig 14B). In comparison to short-term treatment (Fig 6C and 6D), prolonged Baf treatment (for 16 hours) considerably suppressed the phosphorylation of p70S6K (T389) even in nutrient-rich conditions (Fig 14A), indicating that the chronic inhibition of autophagy impaired mTORC1 signaling activity. Prolonged inhibition of autophagy by Baf elevated the ISR, characterized by the increase in its downstream target CHOP (Fig 14C). Since mTORC1 is a central regulator of protein synthesis, I evaluated whether autophagy failure affected the expression of skeletal muscle proteins. Myosin heavy chain (MHC) is one of the most abundant proteins in myofibrils that are responsible for muscle contraction. Indeed, long-term inhibition of autophagy led to a decrease in MHC (Fig 14D), highlighting the vital role of autophagy in preserving the integrity of skeletal muscle proteins. Taken together, persistent defect of autophagy compromises anabolic signaling, induces stress and impairs the synthesis of essential proteins in mouse myotubes.

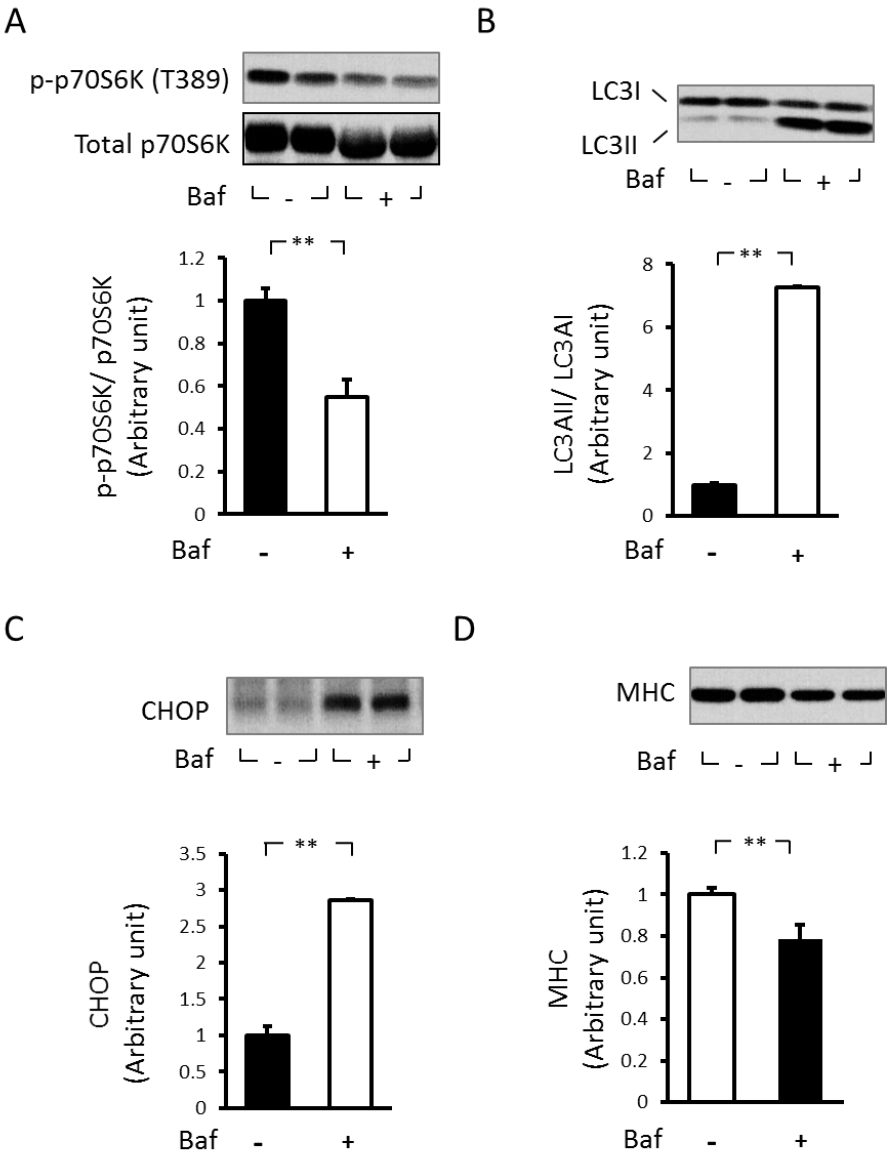


Fig 14. Chronic inhibition of autophagy suppresses mTORC1 signaling and constitutive protein synthesis, and induces the ISR. C2C12 myotubes were treated with or without Baf for 16 hours in basal conditions. Immunoblotting was used to assess the level of (A) phospho-p70S6K (T389)/p70S6K, (B) LC3AII/LC3AI, (C) CHOP, and (D) MHC. Data are expressed as fold of control, and are the mean±SEM for n = 6 samples. **P< 0.01.

4. Discussion

4.1 Autophagy restores mTORC1 signaling during amino acid starvation and prevents the induction of the ISR

mTORC1 tightly coordinates anabolic and catabolic processes in accord with nutritional status especially that of amino acid availability [18, 19, 30]. Autophagy is

under the tight control of mTORC1, which restrains autophagy via ULK1/2-ATG13-FIP200-ATG101 complex and other effectors [83-85]. When nutrients become scarce, mTORC1 loses its negative control over autophagy, allowing this degradation process to be mobilized in order to compensate for the scarcity of intracellular nutrient [83-85]. Until now, the multipronged and delicate regulatory mechanisms by which mTORC1 regulates the process of autophagy have been well-established [63, 64]; and yet, the feedback regulation of mTORC1 by autophagy is less clear. The results of this study demonstrated a feedback loop from autophagy to mTORC1 in C2C12 mouse myotubes. This feedback occurred under amino acid-restricted conditions, as evidenced by the maintenance of mTORC1 signaling during amino acid starvation and its abrogation upon autophagy inhibition (Fig 6C and 6D). This finding reflected the close link between the anabolic mTORC1 pathway and the catabolic process autophagy.

The ISR is a versatile system that can detect a multitude of stresses. Four kinases specifically sense four types of stresses which include amino acid limitation, ER stress, viral infection, and heme deficiency, and the signal converges on the central protein eIF2α, which directly and indirectly modulates cellular processes [94, 99, 101]. Given the sensitivity of ISR and its broad coverage of diverse stresses, studies on this stress response system would provide insight into the nature and consequence of a certain insult. In C2C12 myotubes, amino acid withdrawal did not induce the phosphorylation of eIF2α (Fig 6E), indicating that mouse myotubes are resistant to amino acid deprivation and able to defend against stress. In contrast, the inhibition of autophagy induced the ISR both in the presence and absence of amino acids (Fig 6E). This result suggested that the defect in autophagy disrupts cellular homeostasis and subsequently induces the ISR, and this stress is unlikely to be related to amino acids.

4.2 Autophagy restores mTORC1 signaling which in turn terminates autophagic degradation during prolonged amino acid starvation

Upon amino acid withdrawal, mTORC1 activity and the autophagic flux changed in a temporally associated but opposing manner: mTORC1 signaling initially declined and gradually bounced back afterwards until full recovered, while the activity of autophagy first increased but then diminished (Fig 7A and 7B). By contrast, the ISR was insensitive to amino acid withdrawal but persistently activated under autophagy blockage (Fig 7A). The recovery in mTORC1 and the reduction in autophagy at later hours of amino acid starvation (from 2 to 4 hours) were both abrogated by the inhibition of autophagic degradation (Fig 7A). These data demonstrated that in myotubes autophagy was capable of restoring mTORC1 activity under amino acid deprivation, probably by progressively generating relatively adequate amino acids, which in turn stimulated mTORC1. Previous studies reported that in normal rat kidney cells, mTORC1 signaling activity recovered upon the prolonged deprivation of serum and glutamine (6 to 12 hours of starvation), but it failed to be restored upon the deprivation of serum and all amino acids [144, 225]. In our experimental settings, all the amino acids that are conventionally supplemented during cell culture were deprived, along with serum starvation. Compared to normal rat kidney cells, C2C12 mouse myotubes displayed a remarkable adaptive capacity: mTORC1 signaling was rescued as early as 2 hours after the withdrawal of all amino acids, and it almost resumed full activity at 4 hour of amino acid starvation (Fig 7A). This was fulfilled by the rapid and robust mobilization of the autophagic degradation process in the myotubes (Fig 7A and 7B). The timely restoration of mTORC1 signaling ensures the re-commencement of the normal protein synthesis process and prevents serious protein depletion in myotubes under starvation.

The process of autophagy must be temporally controlled to avoid the degradation of essential cellular components and the exhaustion of proteins. Re-activation of

mTORC1, in addition to resuming the anabolic processes, terminates the flux of autophagy at the end of the 4 hour starvation period (Fig 7A and 7B). This was in concordance with the indiscernible LC3AII accumulation at 4 hour of amino acid deprivation observed previously (Fig 6B). Our study demonstrated that the cessation of autophagy is controlled by mTORC1 which is activated by the feedback of autophagy. Consistent with our results, other studies also showed that the termination of autophagy and the subsequent regeneration of lysosomes were dependent on the degradation of autophagic substrates in the lysosome and the reactivation of mTORC1 [144]. Additionally, the morphology of the lysosomal tubules during ALR was regulated by the mTORC1-mediated phosphorylation of UVRAG and activation of Vps34 [145]. Taken together, mTORC1 controls not only the initiation of autophagy at the early phase but also its termination upon prolonged starvation.

4.3 Autophagy activates mTORC1 by supplying amino acids but regulates the ISR in an amino acid-independent way

Autophagy is a nutrient source that is able to provide amino acids, carbohydrates, lipid, and nucleotides during nutrient restriction [172]. Our results demonstrated that in mouse myotubes autophagy is able to sustain mTORC1 signaling under amino acid limitation through proteolysis and the release of amino acids. First, the re-addition of amino acids resumed mTORC1 activity after amino acid deprivation under autophagy inhibition by CQ (Fig 8A and 8B), suggesting that it was the shortage of intracellular amino acids that inhibited mTORC1 under autophagy inhibition. Second, repletion of intracellular amino acid pool through protein synthesis inhibition was also able to rescue mTORC1 signaling during autophagy inhibition in the absence of amino acid supply (Fig 9A and 9B). This situation mimicked the release of amino acids by autophagy in the cell, and suggested that autophagy modulates intracellular amino acid pool under starvation. The activation of mTORC1 and the repression of autophagy by Chx also indicated that protein synthesis is the principal amino acid-consuming process

that determines amino acid signaling and autophagy is quite sensitive to the consumption of amino acids by this process (Fig 9A, 9B and 9D). Besides, similar to the phenotype under Baf treatment (Fig 6C and 6D), both the lysotropic reagent CQ (Fig 8A and 8B) and the early-stage autophagy inhibitor spautin-1 (Fig 8E) abrogated mTORC1 signaling during amino acid starvation, confirming that the inactivation of mTORC1 by Baf is not due to the inhibition of vATPase. A previous study proposed that after glutamine deprivation, the maintenance of mTORC1 activity was attained by ATF4-mediated upregulation of amino acid transporters and the consequent enhancement of amino acid uptake [225]. However, results from our study showed that autophagy per se is able to generate adequate amino acids to support mTORC1 signaling. Despite the fact that autophagy can also generate carbohydrates, lipids, and nucleotides [172], our study highlighted the capacity of autophagy to yield ample amino acids under starvation, which constitutes a positive signal supporting the anabolic mTORC1 pathway.

On the other hand, the induction of the ISR by autophagy failure is not related to amino acids. This is reinforced by the evidence that amino acid supplementation was unable to alleviate the ISR induced under autophagy inhibition (Fig 8C). Spautin-1 did not induce the ISR (Fig 8F), and it might be explained by the fact that spautin-1 selectively inhibits Vps34-involved macroautophagy [218] but not Vps34-independent microautopahgy and CMA which also mediate stress resistance. The inhibition of protein synthesis dramatically relieved the ISR induced by Baf (Fig 9C), indicating that improved ER homeostasis during reduced production of nascent proteins could prevent the stress under autophagic defect. Taken together, autophagy prevents against the activation of the ISR independent of its role as an amino acid source.

4.4 Autophagy is the primary source of amino acids in mouse myotubes

Compared to the bulk degradation of intracellular components by autophagy, the UPS is a more selective degradation process [186]. Substrate proteins are specifically

modified with the ubiquitin chain, which is mediated sequentially by three enzymes, including ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [186]. Ubiquitinated proteins are recognized and degraded by the proteasome, which processes proteolytic activity. The UPS modulates the abundance and activity of ubiquitin-targeted proteins such as transcription factors and cyclins as a way to control cellular processes and stress responses. In addition, it is also a quality control machinery to clear misfolded or damaged proteins [186]. In certain biological contexts, the UPS has been shown to supply amino acids under starvation [187, 188]. In Hela cells, the UPS was shown to support protein synthesis by providing amino acids in the early hours of amino acid starvation before the activation of autophagy [187]. In yeast and mammalian cells, inhibition of the proteasome led to severe shortage of intracellular amino acids and eventually cell death [188]. However, our results showed that the UPS is not a primary source of amino acids in mouse myotubes. In C2C12 myotubes, inhibition of proteasome with Lac resulted in an accumulation of high molecular weight ubiquitinated proteins (Fig 10A), which were presumably myofibrillar proteins such as myosin. However, the UPS seems to contribute to only a minimal fraction of amino acids in C2C12 myotubes, since proteasome inhibition did not have an appreciable impact on the amino acid-sensitive mTORC1 signaling but autophagy inhibition did under amino acid limitation (Fig 10B). Therefore, the UPS is not an efficient amino acid producer in mouse myotubes. This might be due to the substrate specificity of the UPS system as opposed to the more versatile degradation of proteins, protein aggregates and organelles by autophagy [190]. The recycling of amino acid stores in skeletal muscle is thus largely performed by autophagy under nutrient restriction.

4.5 Autophagy is a vital contributor to amino acid balance in skeletal muscle during nutrient limitation

Skeletal muscle serves as an amino acid store in the body which produces a large amount of amino acids during the post absorptive state or fasting [184]. The free amino acids generated during protein breakdown in skeletal muscle maintains plasma amino acid homeostasis and supports distal metabolic processes such as gluconeogenesis in the liver [185]. The detailed profile of individual amino acids in myotubes under autophagy-proficient or -deficient conditions in the presence or absence of amino acids provided us a clear vision of how autophagy maintains amino acid balance under nutrient limitation (Fig 11). In mouse myotubes, while autophagy had a minimal effect on amino acid profile in nutrient-rich conditions, it contributed greatly to the NEAA pool as well as that of two EAAs threonine and lysine during amino acid restriction (Fig 11A). As to the other EAAs, autophagy had a limited ability to prevent their decline during amino acid deprivation (Table 2), which might be also due to that most of them had been incorporated into *de novo* synthesized proteins by the time of analysis. The overall amino acid profile showed that autophagy, through differentially regulating EAAs versus NEAAs, facilitates in the preservation of intracellular amino acid balance when extracellular amino acid is deficient. Although in liver-specific *ATG7* knockout mice [174] and *ATG5* knockout yeast [226] the contribution of autophagy to individual amino acid levels under starvation has also been delineated, our study illustrated a myotube-specific amino acid profile modulated by autophagy under amino acid limitation. The autophagy-modulated amino acids were overrepresented by NEAAs, threonine and lysine in our study, as opposed to BCAAs in liver autophagy [174] and histidine, methionine, glutamate, and glutamine in yeast [226]. This myotube-specific signature provided clues as to how skeletal muscle sustains amino acid homeostasis and feeds metabolic demand when nutrient is limited under physiological circumstances.

4.6 Autophagy defends against stress

Glucose deficiency affects the glycosylation modification of proteins and thereby disrupts their proper folding, which can induce ER stress [222]. Skeletal muscle cells contain highly abundant and sophisticatedly organized proteins [197], some of which require accurate posttranslational modification. In C2C12 myotubes, deprivation of glucose indeed resulted in the activation of the ISR (Fig 12A and 12B). This was in contrast to the condition of amino acid starvation, which did not induce the ISR (Fig 6E and 7A). Therefore, myotubes seem to be more sensitive to the disturbance of proteostasis induced under glucose limitation than the lack of amino acid supply, probably because in the latter scenario autophagy produces enough amino acids to fend against stress. Although the ISR was activated, autophagy was not induced under glucose starvation (Fig 6A and Fig 12C), indicating that the activation of the ISR system is not sufficient to initiate autophagy in mouse myotubes. Notwithstanding, basal autophagy is still indispensable for stress prevention, since its inhibition aggravated the stress signaling of the ISR under glucose limitation (Fig 12A and 12B).

The function of autophagy is closely linked to the homeostatic status of the cell. Autophagy eliminates cytotoxic components, recycles basic cellular infrastructure and maintains a homeostatic cellular microenvironment. This is particularly important for skeletal muscle, which is prone to accumulate damaged proteins and organelles and susceptible to oxidative stress because of its mechanical activity and high rate of energy production [192, 209, 210]. What's more, decreased activity of autophagy is one pathological factor underlying the aging process in which damage accumulates and cellular function degenerates, and boosting autophagy is a possible means to reverse this process [171]. In our study, the enhancement of autophagic activity (Fig 12E), through the inhibition of its upstream regulator mTORC1 (Fig 12F), significantly repressed the level of the ISR signaling (Fig 12D). Given that the activation of the ISR represents cellular stress and alterations in biological processes, this result supported

the view that the activity of autophagy is positively correlated with cellular homeostasis. Inhibiting mTORC1 is one possible approach to augment autophagy, which can be achieved through caloric restriction or the application of mTORC1 inhibitors, and indeed, both strategies have shown health-improving and lifespan-prolonging effects [171].

4.7 Blockage of autophagy leads to the reprogramming of amino acid metabolism

Cells usually respond to the perturbation of homeostatic state by altering overall amino acid metabolism, and amino acid metabolic genes are enriched in the downstream targets of the ISR system [99]. Our results have shown that inhibition of autophagy activated the ISR in an amino acid-independent manner (Fig 6E and 7A). Consistently, the transcription of genes downstream of the ISR (e.g. *TRIB3* and *SLC7A1*) was increased under autophagy inhibition in both amino acid-rich and amino acid-deprived conditions (Fig 13A). In addition to these ISR-targeted amino acid metabolic genes, other genes involved in the allocation of amine group (e.g. *GPT*, *GOT1*, and *GLUD1*) were also transcriptionally upregulated under the blockage of autophagy, irrespective of amino acid availability (Fig 13B). These results demonstrated that autophagy inhibition disrupts normal amino acid metabolism partially through the ISR pathway. Plausibly, the elevation of these amino acid metabolic proteins serves as an adaptive response to counteract the stress induced under autophagy inhibition. Therefore, the defect in autophagy would reprogram amino acid metabolism and lead to an aberrant amino acid metabolic profile.

4.8 Autophagy is indispensable for skeletal muscle homeostasis

The pronounced phenotype of atrophy in muscle-specific *ATG7* knockout mice manifested the vital importance of autophagy in maintaining protein balance in skeletal muscle [195]. In C2C12 myotubes, under normal nutritional conditions, the acute inhibition of autophagy only had a mild effect on the mTORC1 pathway (Fig 6C and

6D); however, chronic inhibition of this degradation pathway evidently impaired its signaling activity (Fig 14A). Consistent with the activation of ISR under short-term inhibition of autophagy (Fig 6E), prolonged blockage of autophagy resulted in the induction of the ISR downstream target CHOP (Fig 14C). The inhibition of mTORC1 might be attributed to severe amino acid imbalance caused by long-term autophagic defect, given that autophagy regulates the expression of amino acid metabolic genes (Fig 13). More importantly, the level of the muscle protein MHC, which is related to muscle strength and function, was significantly decreased under prolonged inhibition of autophagy (Fig 14D). The low signaling activity of the anabolic mTORC1 pathway (Fig 14A), which promotes protein biosynthesis, may in part account for the reduction in MHC protein (Fig 14D). Thus, autophagy is indispensable for the anabolic synthesis of skeletal muscle proteins, which challenges the traditional view of autophagy as a mere catabolic process. Although excessive protein degradation in skeletal muscle is recognized as one cause of atrophy [202], underactive autophagy can also bring about adverse consequences such as loss of muscle mass and accumulation of damaged organelles as confirmed *in vivo* [195]. During fasting, diminished autophagic degradation did not ameliorate muscle wasting but rather aggravated loss of muscle mass and muscle degeneration [195]. Another instance is sarcopenia, wherein skeletal muscle mass and function decline during aging, associated with a decline in autophagic activity [227] as well as mTORC1 signaling [228, 229]. Autophagy therefore has indirect pro-anabolic functions and positively contributes to protein balance when its activity is within a normal range.

5. Concluding remarks for the first study

In summary, this study demonstrated the differential regulation of the mTORC1 pathway and the ISR system by autophagy in mouse myotubes (the schematic representation of the model is shown in Fig 15). Under amino acid limitation, autophagy potently sustains mTORC1 signaling activity, and this is achieved through

the maintenance of intracellular amino acid balance by autophagy. Moreover, autophagy prevents stress and relieves the ISR signaling, independent of its role as an amino acid supplier. In addition, this study demonstrated that myotubes, which harbor perfuse protein content and potent autophagic capacity, are an ideal in vitro model to study autophagy. Thus, autophagy serves as both an intrinsic self-sustaining system that provides sufficient amino acids to support anabolism under starvation and a stress defense system to maintain homeostasis in skeletal muscle.

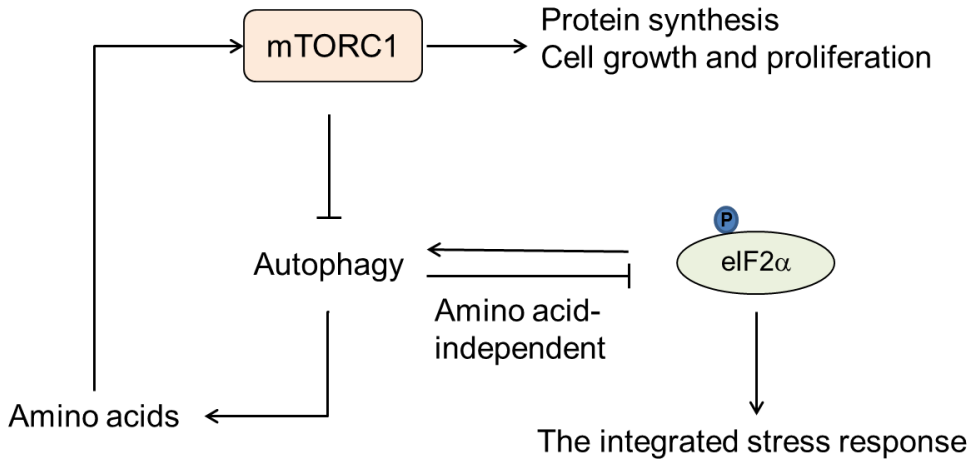


Fig 15. Schematic representation of the proposed model for the regulation of mTORC1 signaling and the ISR by autophagy in mouse myotubes. During amino acid limitation, autophagy is mobilized and it maintains mTORC1 signaling through the release of amino acids; this leads to sustained protein synthesis, cell growth and proliferation. On the other hand, autophagy guards against the ISR in an amino acid-independent manner, which is critical for stress resistance and cellular homeostasis.

Chapter3 The crosstalk between cystine and glutathione in the regulation of amino acid signaling pathways and ferroptosis

1. Preface

The first project delineates how mTORC1 signaling activity is sustained by autophagic nutrient supply under amino acid starvation as well as the prevention of the ISR by autophagy in mouse myotubes. In the human body, the liver and skeletal muscle are two metabolically active tissues that have a global impact on whole body metabolism and physiology. While skeletal muscle is the nutrient reservoir in the body and a highly plastic organ, the liver is the center for systematic metabolic coordination and its function is regulated by nutrients. In the second project, I used a hepatoma cell line (HepG2), and instead of investigating the response to general amino acid starvation, I narrowed my focus on the cell signaling response to the limitation of a unique amino acid, cystine (the oxidized form of cysteine). Cysteine is a special amino acid in that it harbors the redox-active thiol group. Cysteine is also “valuable” as a precursor for the synthesis of glutathione (GSH), the predominant cellular antioxidant. In this study, I attempted to elucidate the regulation of the mTORC1 and ISR pathway by cystine as well as downstream effects, and identify the role of GSH in the cystine-regulated signaling paradigm. Through the mTORC1 and ISR network, I sought to provide insights into how intracellular cysteine deficiency alters cellular processes under physiological and pathological conditions.

2. Introduction

2.1 Cysteine is a unique sulfur amino acid which holds profound biological significance

2.1.1 Cysteine is the only thiol-containing proteinogenic amino acid

In mammals, there are 20 conventional proteinogenic amino acids in which near half of them are essential amino acids that are not synthesized by the cell. The elemental

composition of amino acids is universal, including carbon, oxygen, nitrogen, and hydrogen, with the exception of methionine and cysteine. Methionine and cysteine are the only amino acids that contain the multivalent element sulfur. Although called sulfur amino acids altogether, the chemical and biological natures of the sulfur groups in methionine and cysteine are distinct. While the sulfur group in methionine is relatively inert because of the methyl cap, the naked thiol chain in cysteine that is made of sulfur and hydrogen is chemically and biologically active [230]. The thiol side chain in cysteine is a strong nucleophile and it is susceptible to oxidation, which constitutes the basis for the employment of cysteine as a functional module in various proteins and peptides [231]. In this regards, cysteine is a special and pivotal building block in the biological system.

2.1.2 Metabolism of cysteine

Cystine is the oxidized form of cysteine, formed through the disulfide linkage of two cysteines. Cystine is major supply form such as in the plasma (cystine 50-150 μM versus cysteine 10-25μM) and in cell culture medium because of the oxidizing extracellular milieu, while cysteine is the major form in the cell due to the reducing intracellular microenvironment (cystine usually accounts for less than 10% of total cysteine) [232, 233]. In tissues, cysteine exists in low abundance (10-100 μM) and fluctuates within a narrow range (about 5 folds) in different conditions [232, 234]. A couple of transporters, some with tissue specificity, mediate the uptake of cysteine and cystine [235-238]. Considering that cystine is the prevailing form in the plasma, cystine transporters are considered to account for the majority of cysteine input into the cell. In particular, the cystine/glutamate antiporter (X_c^-)/SLC7A11, which is expressed in most tissues, acts as the primary cystine transporter [239, 240]. System X_c^-, which is composed of a light chain xCT and a heavy chain 4F2 heavy chain (4F2hc) [241], sodium-independently transports cystine in parallel with an equal-molar counter-transport of glutamate, and the concentration gradients of these two amino acids across

the plasma membrane make the import of cysteine favorable [240]. In the cell, the highly reducing intracellular microenvironment converts cystine into cysteine [242]. Apart from exogenous uptake, a few types of tissues are capable of producing cysteine from methionine [243], which is discussed in the following section.

Once imported into the cell, cysteine feeds into a variety of cellular processes. In the anabolic aspect, cysteine is a basic block used for protein synthesis and the biogenesis of GSH (see later section for details) [244]. In the catabolic respect, cysteine can be used to yield Coenzyme A, Taurine, and inorganic sulfur, *etc*.[234]. The fate of cysteine catabolism is determined by a couple of enzymes especially cysteine dioxygenase and desulfhydration-catalyzing enzymes (e.g. cystathionine β-synthase and cystathionine γ-lyase) at the initial step, which shunt cysteine into different catabolic branches [245, 246]. Some of the cysteine-derived molecules such as GSH and taurine have critical biological and physiological functions [244, 247]. Therefore, in addition to its inherent redox property, the importance of cysteine also lies in its contribution to the production of these bioactive biomolecules.

2.1.3 The transsulfuration pathway is a limited source of cysteine

As mentioned above, certain tissues possess the ability to generate cysteine from methionine [243], and it is the reason why this amino acid is classified as a non-essential amino acid. The pathway that converts methionine to cysteine is termed the transsulfuration pathway, which exists predominantly in the liver [244]. This process begins with the donation of methyl group from methionine to adenosine catalyzed by methionine adenosyltransferase (MAT), forming S-adenosyl methionine (SAMe). The methyl group on SAMe is then transferred to methyl receptors, with the former transformed into S-adenosyl homocysteine (SAH). SAH, after the removal of adenosine moiety, is converted to homocysteine. Subsequently, homocysteine is combined with serine by cystathionine β-synthase (CBS) to form cystathionine, and the latter is then converted into cysteine by cystathionine γ-lyase (CGL) [243]. Given

that this pathway is irreversible, the channeling of methionine to cysteine production is a huge expenditure of the cellular methionine pool. The shortage of intracellular cysteine increases the demand for methionine, an essential amino acid that relies exclusively on extracellular provision. Adequate cystine supply spares the consumption of methionine by the transsulfuration pathway and reduces its requirement [248]. Moreover, during prenatal and neonatal phases, intracellular cysteine is absolutely derived from dietary intake since the transsulfuration pathway is defective [249-251]. In addition, extra supplementation of cystine on top of standard dietary intake is required for patients with certain diseases such as infection and cancer, as well as during aging [249, 252, 253]. Of note, in human hepatoma cell line such as HepG2, the activity of MAT which catalyzes the first step of the transsulfuration pathways is absent, entailing the extracellular supply of cystine [235]. Although cysteine is considered a nonessential amino acid, supplementation of this amino acid is essential under certain disease conditions.

2.1.4 Functional roles of cysteine in the cell

Cysteine and cystine are interconvertible through enzyme-independent or -dependent reactions [242]. This conversion lays the foundation for redox reaction, catalytic activity, protein modification and configuration [254]. In proteins, the intrapeptide and interpeptide disulfide bridges are crucial for folding, stabilization, conformational and functional modulation of proteins as well as the formation of multi-unit complexes [230]. In several enzymes, such as protein tyrosine phosphatases (PTPs) and cysteine proteases, the cysteine residue in the catalytic domain initiates the nucleophilic attack towards the substrate, which is usually the first step of reaction [231]. The intracellular redox couples, including GSH/GSSG and thioredoxin reduced/oxidized, together with cysteine/cystine per se, ubiquitously utilize cysteine/cystine as the mediator of oxidoreduction [254]. More importantly, GSH, the major antioxidant defense, requires cysteine for its biosynthesis in the cell [244]. In addition, due to its sensitivity to

oxidation, the cysteine residue in proteins can undergo modification under oxidative stress, such as glutathionylation and oxidation into sulfinic acid, sulfonic acid, or sulfonamide [255], which is always coupled with functional alteration. Additionally, cysteine/cystine per se is increasingly recognized as a redox couple that has a great impact on cell survival and proliferation [256]. Thus, cysteine holds key biological functions either as a constituent of proteins and bioactive peptides or as a free reducing equivalent, making it indispensable for normal cellular processes.

2.2 GSH functions as the predominant antioxidant and a cysteine pool

2.2.1 GSH synthesis requires cysteine

As mentioned above, the cellular requirement of cysteine in part lies in its contribution to the biosynthesis of GSH, an antioxidant present in high abundance (0.5-10 mM) in the cell [257]. GSH is synthesized *de novo* in the cytosol of almost all types of cells from glutamate, cysteine and glycine [244] (Fig 16). This biosynthetic process is accomplished sequentially by two ATP-dependent steps. The first step, which is also the rate-limiting step and catalyzed by glutamate cysteine ligase (GCL), conjugates the γ-carboxylate of glutamate to the α-amino of cysteine. This is then followed by the GSH synthase (GS)-mediated linkage of glycine to γ-glutamylcysteine to generate the tripeptide GSH [244]. GSH production is regulated by conditions such as oxidative stress, dietary protein deprivation, cytokines and hormones at the molecular level, through the modulation of the two enzymes in GSH biosynthesis [244, 257]. Particularly, the rate of GSH synthesis is highly sensitive to the availability of cysteine. The *Km* of GCL for cysteine (0.1-0.3 mM) approximates the normal intracellular cysteine concentration, in comparison to that for glutamate (1.8 mM) which is only 1/10 of the typical cellular glutamate concentration [244]. The intracellular content of GSH would be dramatically impaired when the lack of cellular cysteine persists [258-261]. However, it should be noted that the relationship between cysteine and GSH is

not a one-way traffic, but rather they are reciprocally related and their levels are inter-dependent (Fig 16).

2.2.2 GSH serves as a cysteine reservoir which supplies cysteine via the γ-glutamyl cycle

Not only cysteine determines the level of GSH in the cell, GSH is also a critical modulator of intracellular cysteine pool. GSH is utilized by the cell as a cysteine store, given that intracellular GSH is perfuse whereas cysteine is in low abundance and potentially cytotoxic if raised to supraphysiological levels [234]. The retrieval of cysteine from GSH is performed by the γ-glutamyl cycle [244] (Fig 16). In this cycle, intracellular GSH is exported through specific transporters, and at the extracellular face of plasma membrane, it is degraded sequentially by two ecto-enzymes γ-glutamyl transpeptidase (GGT) and dipeptidase (DP), releasing cysteine which is readily taken up the cell [244]. The activity of γ-glutamyl cycle is enhanced under starvation [261] and oxidative challenge [262]. Nevertheless, its potency in repleting intracellular cysteine under nutrient limitation is controversial [258-261]. *Cho et al.* demonstrated that 24 hours or 48 hours of starvation significantly reduced tissue GSH level without obviously affecting tissue cysteine abundance [259]; however, there is also evidence that tissue cysteine level declined along with GSH reduction during food limitation, indicating that GSH degradation is insufficient to completely compensate for the loss of tissue cysteine under prolonged starvation [258, 260, 261]. Notwithstanding the extent to which GSH repletes cellular cysteine pool, GSH exists as a large reservoir of cysteine in the cell and releases cysteine when needed.

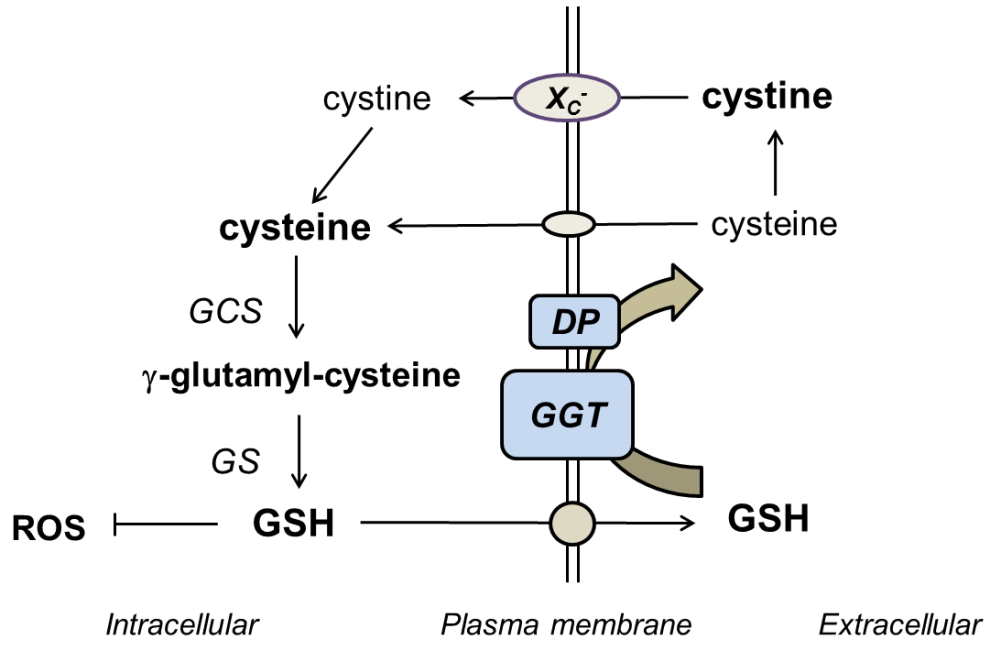


Fig 16. The close interaction between cystine and GSH metabolism. Intracellular cysteine is mainly imported into the cell as its oxidized form cystine, which is readily reduced into cysteine in the cell. In the cytosol, cysteine is used for the synthesis of GSH, which is catalyzed by GCS and GS. GSH can be exported into the extracellular compartment, where it is degraded by two ecto-enzymes GGT and DP, releasing cysteine. Cysteine and its oxidized form cystine can be transported into the cell via specific transporters, contributing to the intracellular cysteine pool. This cycle is termed the γ-glutamyl cycle.

2.2.3 GSH is the predominant cellular antioxidant

GSH gains a great deal of attention as a principal antioxidant, and this facet of functions is extensively investigated. Generally, GSH engages in four antioxidant-related biological processes: neutralization of ROS and other oxidizing molecules, detoxification of electrophiles and xenobiotics, modulation of protein thiols and determination of cellular redox potential [244]. One of the most important functions of GSH as a scavenger of ROS and lipid peroxides is completed via either direct chemical reaction or enzymatic processes [257]. Among these GSH-dependent enzymes, glutathione peroxidases (GPxs) comprise the major defence against a vast diversity of peroxides. Along with the reduction of peroxides, GSH is oxidized into GSSG, which is subsequently reduced to GSH by glutathione reductase (GR) using NADPH as a reducing equivalent [257]. The ROS-eliminating role of GSH is particularly vital to the homeostasis of mitochondria, in which there exists no catalase and is constantly exposed to ROS [263]. In addition, GSH is a primary detoxification agent to eliminate electrophiles and xenobiotics, through conjugation and subsequent extrusion of the derived adduct [244]. Moreover, the abundance of total GSH and the redox state of GSH:GSSG have a global impact on protein thiols [264]. Besides, GSH and GSSG exist as one redox couple which determines cellular redox state and influences various signalling events and cellular processes [264]. Given these critical functions, the loss of GSH would inevitably disrupt cellular redox balance and alter cellular signalling and biological processes if mild, and compromise cell function and viability if severe. Indeed, depletion of GSH usually precedes or occurs during cell death [265, 266].

2.3 The physiological relevance of cysteine

2.3.1 Cysteine is required for growth and development

Mice with genetic deletion of xCT were phenotypically indistinguishable from their wild-type littermate; however, embryoblast cells derived from these knockout mice were unable to survive in normal medium [267]. A more striking phenotype of cysteine

deficiency was observed in GGT knockout mice, which showed pronounced growth retardation, reproductive defects, and premature death at 10 weeks [268, 269]. These defects could be prevented by the supplementation of N-acetyl cysteine (NAC) as a cysteine precursor [269]. What's more, cells isolated from GCS knockout embryos also exhibited markedly cysteine deficiency and required either supplemented GSH or NAC for survival [270]. The phenotype of cysteine deficiency in either GGT [268, 269] or GCS [270] knockout mice highlights the close relationship between GSH metabolism and intracellular cysteine abundance. In humans, significant weight reduction was observed in a unique child case of cystine deficiency which resulted from metabolic defect and diet intervention [271]. Altogether, cysteine is indispensable for growth and proliferation at the cellular level and for tissue development at the systematic level.

2.3.2 The implication of cysteine in cancer

A fascinating feature of cancer cells is that they hijack cysteine to support their vitality and continuous proliferation. Compared to normal cells, higher expression levels of xCT have been observed in a variety of cancers, such as hepatocellular carcinoma [272], ovarian cancers [273], lymphomas [274], gliomas [275], and pancreatic cancers [276], and this has been associated with drug resistance and poor survival. Interestingly, cancer stem cells harness their canonical marker CD44 to stabilize the xCT subunit of cystine transporter, as a means to boost their cystine uptake [277]. The exact reason for cancer's addiction to cysteine remains unclear. One tentative proposition is that cysteine is utilized to produce high level of GSH, as a counterbalance of intrinsic high level of ROS in cancer [278]. Alternative studies also suggested that increased cystine uptake promotes cancer cell survival independent of GSH [279]. To thwart the reliance of cancer on cystine uptake, several drugs targeting xCT (e.g. sulfasalazine and erastin) have been developed and displayed promising anti-cancer potential [276, 280-286]. In certain settings, drug-induced cystine starvation resulted in ferroptosis, a special type of cell death which is morphologically and mechanistically distinct from apoptosis,

autophagy, and necrosis [286]; oxidative challenge, especially elevated lipid peroxidation, is a plausible driver of ferroptotic cell death [286, 287].

In addition to cystine transporter, cancer cells maximize their acquisition of cysteine through γ-glutamyl cycle. GGT, as a key node in the cysteine supplying chain from GSH, is ubiquitously upregulated in a myriad of cancers and correlated with drug resistance and poor prognosis [262]. The augmentation of GGT activity is further facilitated by its asymmetrical distribution in cancer: in contrast to normal tissues which express GGT on their apical surface facing ductal fluid [288], tumor cells have GGT distributed throughout the entire cell membrane, enabling them to reclaim cysteine more efficiently from the GSH in fluids and blood [262]. GGT hyper-activity renders metabolic and growth advantages to cancer cells, especially in nutrient-limited microenvironment; it is also reminiscent of the high demand for cysteine by the proliferative cells.

2.3.3 The implication of cysteine in other pathological conditions

An increasing body of evidence shows that cysteine has extensive implications in health and disease, apart from cancer. Metabolism is one aspect which cysteine impinges upon. Clinical studies have showed that plasma concentration of total cysteine (including cysteine and cystine) is positively correlated with BMI and fat mass, and high plasma total cysteine level is associated with obesity and related metabolic disorders [289]. In addition, high plasma total cysteine is also observed in the diabetic state [290], and its elevation is a feature of chronic kidney diseases in diabetic patients [291-293]. In mice, high cystine intake increased weight gain, reduced metabolic rate, impaired insulin sensitivity and disrupted lipid metabolism [294]. Conversely, cysteine deficiency is a deteriorating factor in the pathogenesis of various diseases. Low plasma cystine levels have been observed in a wide spectrum of diseases, including HIV infection, sepsis, chronic fatigue syndrome, Crohn’s disease, and others, which share common symptoms such as loss of skeletal muscle mass, pronounced decrease in

natural killer (NK) cell function, and increased urea production [295]. Although controversial [296], scientists even proposed cysteine deficiency as a driving force in the aging process [253, 297-299]. In this regard, supplementation with cysteine derivatives or cysteine-rich proteins represents a potential anti-aging regime that can combat some of the age-related degenerative complications [253, 298-300]. In addition, cysteine supplementation is also a promising adjuvant therapy to ameliorate some pathological conditions, and it showed beneficial effects such as attenuating muscle fatigue [301, 302], improving insulin sensitivity [303, 304], preventing oxidative damage [304, 305], and suppressing inflammatory responses [306]. All the *in vivo* and clinical evidence draw attention to the nonessential amino acid cysteine as an essential participant regulating a variety of biological and physiological processes.

2.4 Rationale and hypothesis of this study

The existing plethora of evidence points to the profound significance of cysteine in biological and physiological processes. However, current knowledge on the regulation of cell signaling and biological processes by cysteine is relatively lacking, impeding the understanding of the cysteine-involved pathological conditions. In addition, given that cysteine is the crucial precursor for GSH synthesis, it is tempting to posit that the alteration of GSH content and the consequent disturbance of redox homeostasis is a causal factor for the abnormal phenotypes under cysteine deficiency. This study attempted to address how cystine regulates cell signaling, with a focus on two nutrient- and stress-sensitive pathways, mTORC1 signaling and the ISR. mTORC1 signaling, as a pro-anabolic pathway, is well-known to be amino acid dependent; however, until now this list of amino acids mainly include essential amino acids and glutamine [1, 28-30, 33-36]. The ISR senses a wide variety of stresses including amino acid limitation, and mediates a comprehensive adaptive response. Moreover, both signaling pathways are redox-sensitive [60, 61, 307]. The regulation of these two pathways by cystine availability was investigated in HepG2 cells which exclusively depend on extracellular

cystine as a source of cysteine, and determined whether and how GSH participates in this regulatory framework. The chronic effects of cystine deprivation on cell viability, cell growth and cell proliferation were also investigated. Through the delineation of the signaling response to cystine starvation, I aim to shed light on the mechanism by which cysteine deficiency affects normal physiological functions and the role of GSH in this process.

3. Materials and Methods

3.1 Materials

Rapamycin (Rapa) was purchased from Tocris Bioscience (Bristol, United Kingdom). Cycloheximide (Chx), tunicamycin (Tm), 6-diazo-5-oxo-L-norleucine (DON), L-glutathione reduced (GSH), glutathione reduced ethyl ester (GSHee), N-acetyl-L-cysteine (NAC), antimycin A (AMA) and phenylarsine oxide (PAO) were from Sigma-Aldrich (St. Louris, MO, USA). OU749, buthionine sulfoximine (BSO), sulfasalazine (SAS), and ferrostatin-1 (Fer-1) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). Antibodies specific for p70S6 kinase, phospho-p70S6 kinase (Thr389), S6 ribosomal protein, phospho-S6 ribosomal protein (Ser235/236), LC3B, eIF2α, phospho-eIF2α (Ser51), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), p62, PERK, Bip, mTOR, and Raptor were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific for phospho-GCN2 (Thr899) was from Abcam (Cambridge, United Kingdom). ATF4 antibody was from Santa Cruz Biotechnology, Inc (Dallas, Texas, USA). Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). For co-immunoprecipitation, IP antibody for mTOR and protein A agarose beads were from Cell Signaling Technology.

3.2 Cell Culture and Cell Treatment

Hepatoma cells HepG2 and Huh7 were maintained in growth medium (high glucose DMEM from PAA Laboratories, GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in the presence of 5% CO2.

Cells were plated in 6-well plate 24 hours before treatment. Unless otherwise indicated, cell treatment was performed in basal medium or cystine-free medium. Basal medium was prepared by supplementing DMEM (Gibco, Carlsbad, CA, USA, no cystine, no methionine, and no glutamine) with 100 µM methionine, 4 mM glutamine, 1 mM sodium pyruvate, 0.2% bovine serum albumin, 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µM cystine. Cystine-free medium was prepared in the same way as basal medium, except that 100 µM cystine was excluded. For cystine re-stimulation, 200 µM cystine was added to cystine-free medium. For cell viability assays, medium was supplemented with 10% dialyzed FBS.

For GSH assay, cells were treated in Earle's balanced salt solution (EBSS), supplemented with 25 mM glucose, 4 mM glutamine, 1×MEM vitamin solution (Life technologies, Carlsbad, CA, USA), 0.22% wt/vol NaHCO3, 1 mM sodium pyruvate, 0.2% bovine serum albumin, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. Cystine-free amino acid solution was prepared as 50× stock solution, according to the formula of 50×MEM amino acid solution from Life technologies with cystine excluded. When used, stock was added to the medium to a final 1× concentration. 100 µM cystine was added to the medium for cystine-supplemented conditions.

For the measurement of reactive oxygen species and lipid peroxidation, cells were treated in DMEM medium without phenol red. This medium formulated according to high glucose DMEM (PAA Laboratories, GE Healthcare, with 1 mM sodium pyruvate, 0.22% wt/vol NaHCO3, 4 mM glutamine, 25 mM glucose), except that cystine was

excluded for cystine starvation conditions. 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% dialyzed FBS (Gibco) were supplemented to the medium.

The following compounds were used in this study: 20 nM Rapa was used to inhibit mTORC1 activity; 2.5 µM Chx was utilized to inhibit protein synthesis; 1 µg/ml or 10 µg/ml Tm was applied to induce the unfolded protein response; 1 mM GSH, 5 mM NAC and 5 mM GSHee were supplemented as indicated; 250 µM OU749 or 1 mM DON was used to inhibit the ectoezymatic activity of GGT; 300 µM BSO was applied to the cell to deplete intracellular GSH; 20 µM AMA was utilized to increase ROS and deplete GSH in the cell; the Xc- inhibitor 0.5mM SAS was administered to the cell to induce oxidative stress; PAO (5 µM) was utilized as a strong thiol oxidizer; 1 µM Fer-1 was used as an inhibitor of ferroptosis.

3.3 Immunoblot

HepG2 cells in 6-well plate were lysed on ice with 200 µl RIPA buffer [25 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1% vol/vol IGEPAL, 1 % wt/vol sodium deoxycholate, and 0.1% wt/vol SDS], supplemented with 5 mM β-glycerophosphate, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 5 mM sodium pyrophosphate, and protease inhibitor cocktail (Pierce, Thermo Scientific, Waltham, MA, USA). Cell lysate was subjected to sonication and centrifuged at 14, 000 rpm at 4°C to remove cell debris. The total protein content of homogenate was determined using Pierce BCA protein assay kit (Thermo Scientific). Proteins were denatured in sample buffer [32.5 mM Tris·HCl (pH 6.8), 2.5% vol/vol glycerol, 1% wt/vol SDS, 0.005% wt/vol bromophenol blue, and 50mM dithiothreitol] and heated for 10min at 65 °C. Proteins were then separated by 10% or 7.5% SDS-PAGE, and transferred to Immun-Blot PVDF membrane (BioRad Laboratories, Hercules, CA, USA). Membranes were blocked with 5% skimmed milk in Tris·buffered saline containing 0.1% Tween 20 (TBST) for 1 hour at room temperature, and then incubated with respective primary antibodies overnight at 4°C. HRP-conjugated secondary antibody was used to detect

the primary antibodies, and ECL method (Pierce, Themo Scientific) was used to generate the chemiluminescence signal.

3.4 Immunoprecipitation

Treated cells (in 100 mm culture dish) were washed with cold PBS and lysed in cold cell lysis buffer [10mM KPO4 (pH 7.2), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM beta-glycerophosphate, 1 mM sodium orthovanadate (Na3VO4), 0.3% CHAPS, and 1X Protease inhibitor cocktail]. Lysate was collected in 1.5 ml Eppendorf tubes and incubated for 1 hour at 4°C with gentle rotation. Then lysate was subject to centrifugation, and the supernatant was collected for protein quantification. The protein in whole cell lysate was diluted to 1.5 µg/ µl, and separated into two portions which are used as for immunoprecipitation and as whole cell lysate respectively. Antibody for immunoprecipitation was added to the 200 µl lysate at 1:40 ratio (v/v), followed by incubation overnight at 4°C. Cell lysate mixture was then incubated with protein agarose A beads for 3 hours at 4°C and gently washed three times with cell lysis buffer. The pellet was denatured in sample buffer [32.5 mM Tris·HCl (pH 6.8), 2.5% vol/vol glycerol, 1% wt/vol SDS, 0.005% wt/vol bromophenol blue, and 50mM dithiothreitol] for 5 min at 100°C. After centrifugation, the supernatant was loaded into SDS-PAGE gel. Immunoblot was performed as described above.

3.5 Quantitative Real-time PCR

HepG2 cells in 6-well plates were treated as indicated. RNA was purified using ReliaPrep RNA Cell Miniprep System (Promega, Madison, WI, USA) according to the manufacturer's recommendations, and 1.5 µg total RNA was subjected to reverse transcription using the ImProm-II Reverse Transcription System (Promega). Real-time PCR was performed on ABI 7300 Real-Time PCR System (Life Technologies) using SYBR Green Select Master Mix (Applied Biosystems, Carlsbad, CA, USA). The primer sequences for genes analyzed in this study are listed in Table 3. Relative

quantitative method was applied for data analysis, by normalizing the mRNA level of all genes against that of the housekeeping gene β-actin.

Table 3. Primer sequences for real-time PCR in the second study

Target gene	Description	Primer sequence		GeneBank accession No.
		FWD (5’ to 3’)	REV (5’ to 3’)	
TRIB3	Homo sapiens tribbles pseudokinase 3	TGATCTCAAGCTGTGTCGCT	CTGCCTTGCCCGAGTATGAG	NM_001301188
SLC7A11	Homo sapiens solute carrier family 7 (anionic amino acid transporter light chain, xc- system), member 11	AAGCACACTCCTCTACCAGC	AGTGGCACCTTGAAAGGACG	NM_014331
SLC7A1	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	CTGTCTGTTCGCGATCCTGC	TTCAGAGCTGGAATATGACGGG	NM_003045
SLC38A2	Homo sapiens solute carrier family 38, member 2	TGCTCTGAAAAGCCATTATGCAG	CCAAGGATTCCACTGCCCAC	NM_018976
ACTB	Homo sapiens actin, beta	TGGATCAGCAAGCAGGAGTATG	GCATTTGCGGTGGACGAT	NM_001101

3.6 GSH Assay

Cells in 6-well plate were treated in EBSS medium as desired. After treatment, medium was collected for the determination of extracellular total GSH level, while cells were lysed in 200 µl cold 5% 5-sulfosalicylic acid dihydrate and the supernatant was collected after centrifugation for the measurement of intracellular total GSH. The pellet was reconstituted in 100 µl RIPA buffer to dissolve proteins, and the content of proteins was determined using Pierce BCA protein assay kit (Thermo Scientific). The level of GSH was measured using Glutathione Colorimetric Detection Kit (Arbor Assays, Ann Arbor, Michigan, USA) according to the manufacturer's instructions. The concentration of intracellular GSH was normalized to the intracellular protein content. Data were presented as nmol GSH/µg protein for intracellular GSH, and nmol for extracellular GSH.

3.7 Measurement of Reactive Oxygen Species and Lipid Peroxidation

Cells were treated in DMEM medium without phenol red (with 10% dialyzed FBS) for desired durations. To determine the level of lipid peroxidation, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY 581/591 C11, Life technologies) was added to the medium to a final concentration of 5 µM after the treatment. Alternatively, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA, Life technologies) was applied to a final concentration of 5 µM for reactive oxygen species measurement. Cells were incubated with the dye in the dark for 30 minutes at 37°C. Then cells were harvested by trypsinization, washed once with phosphate-buffered saline (PBS), and re-suspended in 300 µl DMEM medium without phenol red supplemented with 0.2% bovine serum albumin (without dialyzed FBS). Fluorescence was analyzed using a flow cytometer (BD FACSCanto™ II, BD Biosciences, Franklin Lakes, NJ, USA) equipped with a 488 nm laser for excitation. Data were collected using the 530/30 nm band-pass filter, with 10,000 cells per sample. Single cells were gated using the FSC-

height vs FSC-area dot plot, and data was analyzed using FlowJo software (Tree Star Inc, Oregon, USA).

3.8 Analysis of Cell Size and Cell Cycle

After treatment, cells in 6-well plate were trypsinized, washed once with PBS, and re-suspended in 100 µl PBS supplemented with 1% fetal bovine serum. Subsequently, cells were fixed with 1 ml 70% ethanol at -20 °C for 20 min. Ethanol was removed by centrifugation, and fixed cells were washed once with PBS supplemented with 1% fetal bovine serum. Then cells were re-suspended in 0.5 ml FxCycle PI/RNase staining Solution (Life technologies) and incubated at 37 °C for 45min in the dark. Stained samples were subjected to FACS analysis using BD FACSCanto™ II flow cytometer, with 488 nm excitation laser and 585/42 nm band pass filter. 10,000 events were collected for each sample. Single cells were gated away from aggregates using an FSC-width versus FSC-area dot plot, and the cell cycle was analyzed using FlowJo software (Tree Star Inc, Oregon, USA). The mean FSC-H of G1 phase cells was analyzed as a measurement of cell size.

3.9 MTT Assay

HepG2 cells were treated as indicated in 96-well plate for 48 hours. MTT assay was used to measure cell viability. Briefly, MTT was added to each well to a final concentration of 5 mg/ml, followed by incubation for 4 hours at 37°C in the dark. After medium was removed, DMSO was added to each well to dissolve the formazan crystals, and absorbance at 540 nm was measured with a microplate reader (Infinite M200, Tecan Group Ltd., Männedorf, Switzerland).

3.10 Determination of Cell Death by PI Staining and Flow Cytometry

After 48 hours of treatment, cells were trypsinized, washed once with PBS, and then suspended in 300 µl Dulbecco's phosphate buffered saline (DPBS) supplemented with 0.2% bovine serum albumin containing 0.3 µg/ml propidium iodine (PI, Sigma-

Aldrich). After 30 minutes of incubation at 37°C in the dark, the percentage of PI positive cells were analyzed using a flow cytometer (BD FACSCanto™ II, BD Biosciences), with 488 nm excitation laser and 585/42 nm band pass filter. 10,000 events per sample were collected, and the percentage of dead cells was presented as % of PI positive cells.

3.11 Statistical Analysis

Data are presented as mean± SEM or mean± SD for at least 3 independent replicates. Differences among groups were determined by one-way ANOVA followed by Fisher's least significant differences *post hoc* analysis. Significance was accepted at $p < 0.05$.

4. Results

4.1 Cystine availability regulates mTORC1 signaling and the ISR

Although it is well-established that mTORC1 pathway depends on the presence of essential amino acids as well as glutamine to maintain its anabolic signalling [1, 28-30, 33-36], the role of cysteine is unclear. Cysteine exists as the main intracellular form while cystine is the predominant form in the plasma and used in cell culture [232]. To deplete intracellular cysteine, I removed cystine supply to HepG2 cells, which are defective in the transsulfuration pathway and thus unable to convert methionine to cysteine [235]. Cystine limitation led to a pronounced time-dependent suppression of mTORC1 signaling activity, as demonstrated by the gradual decrease in the phosphorylation of p70S6K (T389) and S6 (S235/236), the major signaling axis downstream of mTORC1 (Fig 17A). In addition, I also observed a dynamic conversion from LC3B I to II, and a gradual decrease in p62, both of which indicated the mobilization of autophagy (Fig 17A). In comparison, ERK phosphorylation remained largely unchanged after cystine deprivation (Fig 17A), which suggested that it did not mediate the phosphorylation of S6.

The ISR is a versatile system that senses a vast spectrum of stresses and mediates cellular adaptation [94]. Four upstream kinases are activated separately by four types of stresses, namely, GCN2 for amino acid limitation, PERK for ER stress, PKR for viral infection, and HRI for heme deficiency [94]. I speculated that the restriction of cystine might induce cellular stress which signals to the ISR system. Supporting this speculation, cystine deprivation acutely stimulated the phosphorylation of eIF2α at S51, which is the central node in the ISR signaling network, together with the upregulation of ATF4, the downstream effector of eIF2α (Fig 17A). The response of the ISR to cystine starvation occurred as early as 1 hour, and persisted throughout the whole starvation period (Fig 17A), which demonstrated that the ISR is a rapid and stable stress response under cystine limitation.

The inhibition of mTORC1 signaling and the activation of the ISR by cystine starvation were reversible: 3 hours of cystine re-supplementation was able to restore mTORC1 signaling and suppress the ISR after 6 hours of cystine deprivation, as shown by the recovery in the phosphorylation of p70S6K (T389) and S6 (S235/236) and the reduction of phospho-eIF2α (S51) and ATF4 (Fig 17B). Treatment of the mTORC1 inhibitor rapamycin (Rapa), as expected, abolished the reactivation of mTORC1 signaling by cystine, confirming the regulation of this complex by cystine (Fig 17B). In contrast, Rapa did not have any discernible effects on either eIF2α phosphorylation or ATF4 abundance (Fig 17B), ruling out the signaling communication from mTORC1 pathway to the ISR system. The regulation of mTORC1 signaling by cystine availability was also observed in Huh 7 cells, in which cystine re-supplementation elevated phospho-p70S6K (T389) and phospho-S6 (S235/236) and these effects were abolished by Rapa (Fig 17C). NAC is a membrane-permeable derivative of cysteine, which has wide clinical applications for the purpose of subduing inflammation and oxidative damage as well as detoxification [308]. Under cystine deprivation, NAC supplementation was capable of preserving mTORC1 signaling activity (Fig 17D),

demonstrating the efficiency of this compound in repleting cysteine and the regulation of mTORC1 by cysteine in the cell.

To exclude the possibility that the signaling effects induced by cystine starvation are a result of the secondary methionine depletion, cells were supplied with twice the physiological concentration of methionine in the absence of cystine. However, excess methionine could not compensate for the cystine limitation-induced effects on mTORC1 and the ISR (Fig 17E). The depletion of methionine on its own as an essential amino acid inactivated mTORC1 (Fig 17E), but it did not activate eIF2α (Fig 17E), indicating that the ISR was more sensitive to the shortage of cysteine than that of methionine in the cell. These results are consistent with the notion that HepG2, as well as other hepatoma cell lines, are unable to convert methionine to cystine [235]. Hence, the reduction of intracellular cysteine accounts for the inhibition of mTORC1 signaling and the activation of the ISR in HepG2 cells.

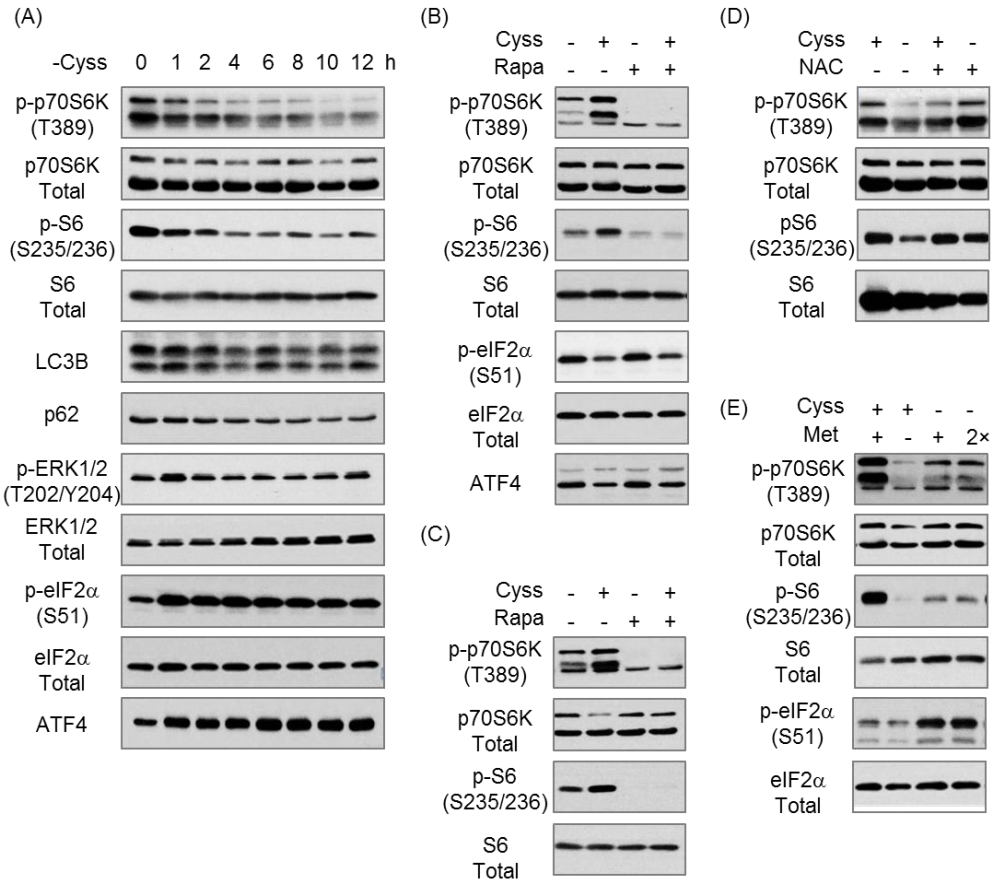


Fig 17. Cystine availability regulates mTORC1 signaling and the ISR. (A) HepG2 cells were deprived of cystine for various durations. (B) HepG2 cells were cystine-starved for 6 hours before being stimulated with or without cystine (200 μM) for 3 hours. Rapa (20 nM) was added to the medium 1 hour prior to cystine stimulation. (C) Huh7 cells were cystine-starved for 1 hour before being stimulated with or without cystine (200 μM) in the presence or absence of Rapa (20 nM) for 2 hours. (D) HepG2 cells were treated with or without NAC (5 mM) in the presence or absence of cystine (100 μM) for 3 hours. (E) HepG2 cells were treated with or without cystine (100 μM) in the presence or absence of methionine (101.4 μM) for 9 hours. Twice the normal concentration of methionine (202.8 μM) was supplemented to the indicated groups (2×). Immunoblotting was conducted to assess the levels of phosphorylated and total proteins in mTORC1 pathway and the ISR system.

4.2 Cystine deprivation disrupts GSH homeostasis

Given that cysteine is an essential precursor for the synthesis of GSH [244], I sought to determine whether GSH abundance is affected by cystine deprivation. Deprivation of cystine resulted in a progressive decline in the level of intracellular total GSH (including GSH and GSSG, hereafter referred to as GSH), to almost one third of the normal level by 9 hours of cystine starvation (Fig 18A, left panel). In parallel, there was a gradual accumulation of GSH in the extracellular milieu (Fig 18A, right panel), indicating an increase in its export. The mild accumulation of GSH in the extracellular space during the early hours was due to its degradation, since inhibition of GGT by 6-diazo-5-oxo-L-norleucine (DON) the buildup of extracellular GSH became more evident at 2 hours of cystine limitation (Fig 18B). When the deprivation of total amino acids and that of cystine were compared, both drove the decline in intracellular GSH and the increase in its export to a similar extent (Fig 18C). However, the turnover of GSH seemed to be particularly sensitive to cystine availability, as the supply of cystine alone in the absence of the other amino acids was sufficient to prohibit the alteration in GSH level both intracellularly and extracellularly (Fig 18C). The enhancement of GSH export and its degradation reflects an adaptive response of the cell to cystine limitation, as a means to promote the reclamation of cysteine from GSH via the γ-glutamyl cycle [244]. Altogether, cystine limitation dramatically disrupts GSH homeostasis, characterized by a significant depletion of intracellular GSH content and an acceleration of its export.

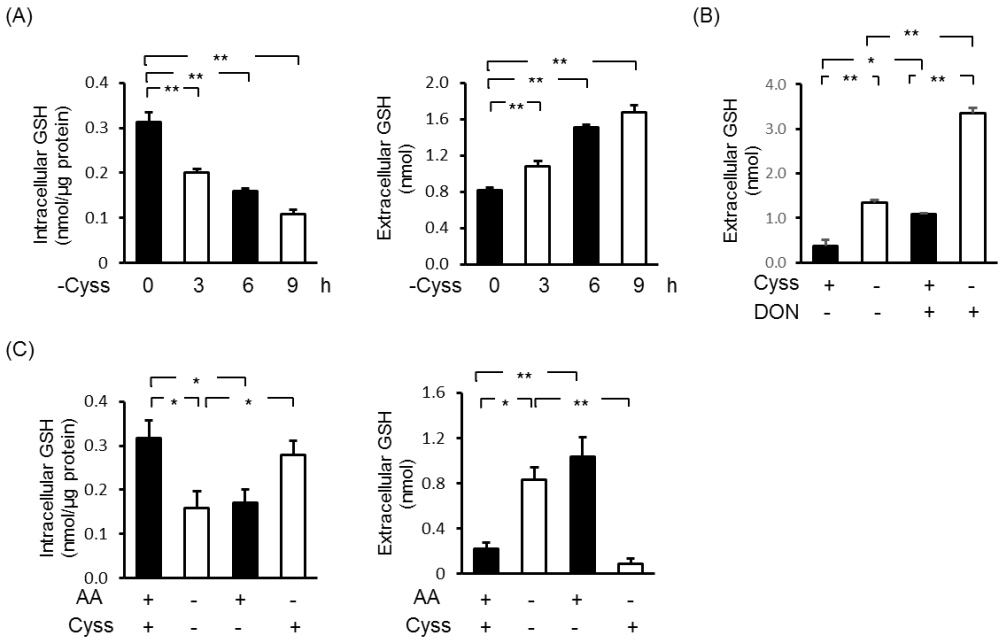


Fig 18. Cystine availability regulates GSH homeostasis. (A) HepG2 cells were cystine-starved in EBSS medium for various durations, and intracellular (left panel) and extracellular GSH (right panel) were measured. (B) HepG2 cells were treated in EBSS with or without DON (1 mM) in the presence or absence of cystine (100 μM) for 2 hours. The level of extracellular GSH was measured. (C) HepG2 cells were treated in EBSS with or without cystine (100 μM) in the presence or absence of cystine-free amino acids (formulated according to MEM amino acid solution from Life technologies) for 8 hours. The levels of intracellular (left panel) and extracellular (right panel) GSH were determined. Data are expressed as mean±SEM for n = 3-6 samples. *P<0.05; **P< 0.01.

4.3 Cystine starvation induces AAR without perturbing redox balance

GSH is the predominant antioxidant defence against oxidative stress in the cell [244]; therefore, the depletion of intracellular GSH under cystine starvation may induce redox imbalance dominated by ROS. Intracellular soluble and lipid ROS were evaluated using CM-H2DCFDA and BODIPY 581/591 C11, respectively. Unexpectedly, the level of intracellular ROS did not increase after cystine deprivation (Fig 19A). Also, neither short-term (Fig 19B) nor long-term (Fig 19C) cystine deprivation induced lipid peroxidation. In contrast, SAS, which is a xCT inhibitor that has been confirmed to induce oxidative stress [309], elevated both intracellular soluble ROS (Fig 19A) and lipid ROS levels (Fig 19B and 19C). It indicated that cystine limitation does not elicit oxidative stress in the cell.

GSH has been proposed as a redox buffer involved in disulphide formation and protein folding in the ER [310]. Next I assessed whether ER stress was induced under cystine deprivation. The signals of ER stress and AA limitation stress are transduced to eIF2α through PERK and GCN2 respectively [94], making them specific markers for these two stresses. Under cystine starvation, phosphorylation of PERK, as assessed by the mobility shift of this protein, remained largely unaltered, whereas tunicamycin (Tm), the canonical inducer of ER stress, significantly retarded PERK mobility indicative of its increased phosphorylation (Fig 19D). Consistently, Bip, as a downstream target of UPR [311], was dose-dependently upregulated by Tm but not by cystine limitation (Fig 19D). In contrast, phospho-GCN2 (T899) was significantly induced by cystine deprivation but not by Tm (Fig 19D), indicating that the AAR which signals through the GCN2-eIF2α-ATF4 axis was induced under cystine limitation. Thus, cystine starvation probably induced a stress similar to total amino acid limitation by nature.

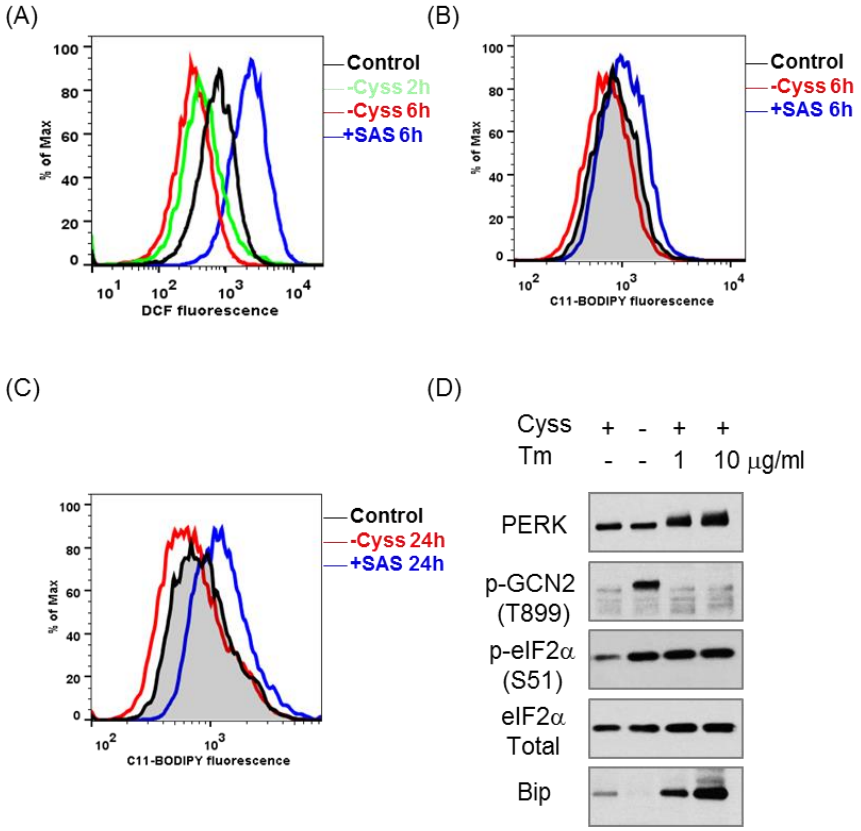


Fig 19. Cystine deprivation induces the AAR without disrupting redox balance and ER homeostasis. (A) HepG2 cells were deprived of cystine for 2 and 6 hours, or treated with SAS (0.5 mM) for 6 hours. Soluble ROS were assessed by flow cytometry using CM-H2DCFDA. Representative result for 3 independent experiments is shown. (B, C) HepG2 were deprived of cystine for 6 hours (B) or 24 hours (C), or treated with SAS (0.5 mM) for for 6 hours (B) or 24 hours (C). Lipid ROS were assessed by flow cytometry using BODIPY 581/591 C11. Representative result for 3 independent experiments is shown. (D) HepG2 cells were treated with or without Tm (1 μg/ml or 10 μg/ml) in the presence or absence of cystine (100 μM) for 9 hours. Immunoblotting was performed to evaluate the levels of phosphorylated and total proteins in mTORC1 pathway and the ISR system.

4.4 GSH rescues mTORC1 signaling and the ISR under cystine starvation in a GGT-dependent manner

Given the decline in intracellular GSH under cystine starvation, I next sought to determine whether the effects on mTORC1 and ISR pathways were mediated by this crucial antioxidant. Cells were supplemented with GSH under cystine starvation, and the results showed that GSH not only effectively rescued the phosphorylation of p70S6K (T389) and S6 (S235/236) in the mTORC1 pathway, but also completely suppressed the induction of phospho-GCN2 (T899), phospho-eIF2α (S51), and ATF4 in the ISR system (Fig 20A). However, it should be noted that GSH can act as a supplier of cysteine through its extracellular degradation [244], so the effects of GSH supplementation on these two pathways could be mediated by GSH per se or resulted from the secondary effect of GSH-released cysteine. To identify which is the intermediate regulator under this scenario, ecto-degradation of GSH was inhibited using a specific GGT inhibitor, OU749 [312]. Surprisingly, OU749 completely abolished the rescue effects of mTORC1 signaling and the ISR by GSH under cystine starvation (Fig 20A), suggesting that the degradation of GSH is required for its protective effects on these two pathways.

There is still debate over whether GSH can be readily taken up by the hepatocytes [313]. To circumvent this issue, I utilized a membrane-permeable counterpart of GSH, GSHee. Strikingly, GSHee regulated mTORC1 and the ISR in a similar manner as GSH: under cystine starvation, GSHee recovered mTORC1 activity, although to a lesser extent than GSH, and it relieved the ISR; however, both were abrogated upon the treatment of OU749 (Fig 20B). Therefore, GSH per se is not a regulator of mTORC1 and ISR pathways; it indirectly modulates these two pathways through the release of cysteine during its degradation.

To assess the contribution of endogenous GSH under cystine starvation, I depleted intracellular GSH using BSO, which inhibits GSH *de novo* synthesis. Prolonged

treatment with BSO considerably decreased the level of intracellular GSH, to less than 10% of that in control cells, both in the presence and absence of cystine (Fig 20C). Depletion of endogenous GSH evidently exacerbated the suppression of mTORC1 signaling under cystine deprivation, evidenced by more striking decline of phospho-p70S6K (T389) and phospho-S6 (S235/236) (Fig 20D). In contrast to mTORC1 pathway, BSO did not affect the response of the ISR system to cystine starvation (Fig 20D), indicating that this aspect of stress response is sensitive to but not dose-dependent on the level of cysteine in the cell. Notably, in the presence of cystine supply, GSH depletion by BSO alone hardly had any discernible effect on either the mTORC1 pathway or the ISR system (Fig 20D), reinforcing our hypothesis that GSH per se is not a regulator of these two pathways. Hence, endogenous GSH protects against the suppression of mTORC1 signaling under cystine limitation by virtue of supplying cysteine.

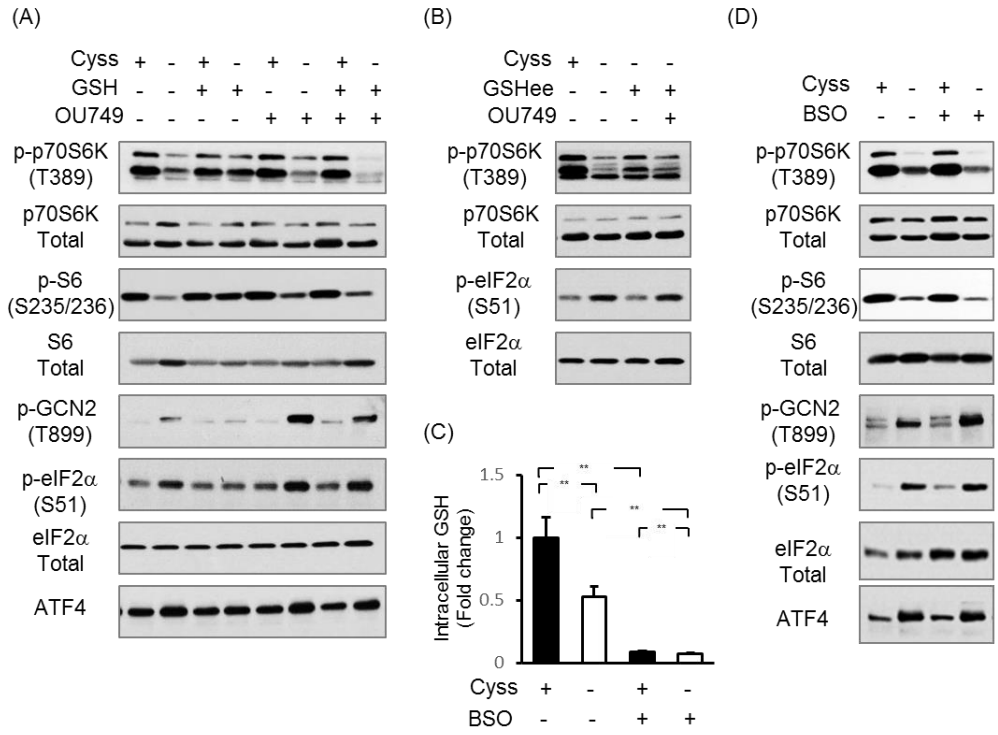


Fig 20. GSH regulates mTORC1 signaling and the ISR under cystine starvation in a GGT-dependent manner. (A) HepG2 cells were treated with or without OU749 (250 μM) in the presence or absence of cystine (100 μM) for 9 hours. GSH (1 mM) was supplemented during the final 3 hours of treatment. (B) HepG2 cells were treated with or without OU749 (250 μM) in the presence or absence of cystine (100 μM) for 9 hours. GSHee (5 mM) was supplemented during the final 3 hours of treatment. (C) HepG2 cells were pretreated with or without BSO (300 μM) for 18 hours, and then treated with or without BSO (300 μM) in the presence or absence of cystine (100 μM) for 9 hours. Intracellular GSH was measured. Data are expressed as mean±SEM for n = 5 samples. **P< 0.01. (D) HepG2 cells were pretreated with or without BSO (300 μM) for 18 hours, and then treated with or without BSO (300 μM) in the presence or absence of cystine (100 μM) for 4 hours. Immunoblotting was performed to evaluate the levels of phosphorylated and total proteins in mTORC1 pathway and the ISR system.

4.5 GSH prevents the transcriptional alteration of amino acid metabolic genes under cystine starvation

Upon activation under stress, the ISR system reprograms cellular metabolism and cell viability via ATF4-mediated transcriptional regulation of CARE-containing genes [102]. In the specific context of AAR, the signaling through GCN2-eIF2α-ATF4 targets a set of genes containing a specialized CARE motif termed AARE [102]. Our previous data confirmed that cystine starvation activated the AAR branch of the ISR network, herein I assessed the transcription of several AAR downstream genes, including *TRIB3*, *SLC7A11*, *SLC7A1*, and *SLC38A2* [314, 315]. Consistently, the mRNA levels of *TRIB3*, *SLC7A11*, *SLC7A1*, and *SLC38A2* displayed a similar pattern under the experimental conditions: while they were upregulated by cystine deprivation, supplementation of GSH prevented their transcriptional induction under cystine deprivation; however, this suppressive effect by GSH was abrogated by OU749 (Fig 21). The transcriptional profile of these downstream genes (Fig 21) is consistent with the signaling activity of the ISR at the protein level (Fig 20A). Thus, cystine and GSH modulate the transcriptional program downstream of the ISR in an interdependent manner.

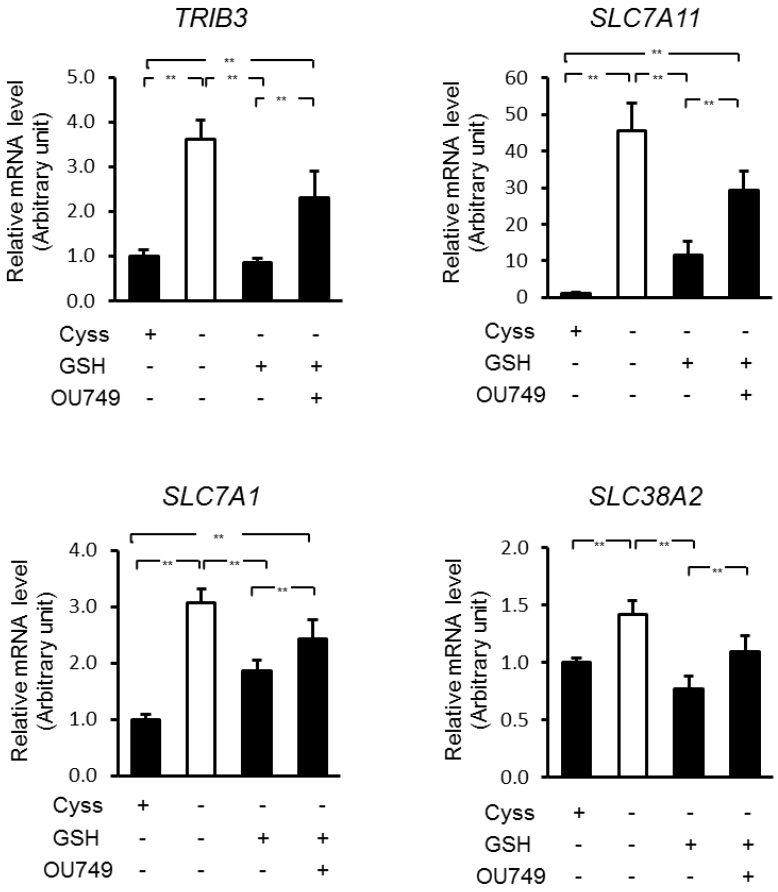


Fig 21. Cystine and GSH regulate the transcription of the ISR downstream genes. HepG2 cells were treated with or without OU749 (250 μM) in the presence or absence of cystine (100 μM) for 9 hours, and GSH (1 mM) was supplemented during the final 3 hours of treatment. The mRNA level of target genes was analyzed by real-time PCR and normalized against that of β-actin. Data are expressed as fold of control. Data are the mean±SD for n = 6 samples. **P< 0.01.

4.6 Inhibition of protein synthesis rescues both mTORC1 and the ISR during cystine starvation

Protein synthesis is prioritized over other cysteine-consuming biological processes under normal conditions and particularly under sulfur-deficient circumstances [316]. This may explain why the large amount of endogenous GSH failed to prevent the decline in intracellular free cysteine and the consequent stresses under cystine deprivation (Fig 20D). The majority of available cysteine, including the fraction contributed by GSH, may be incorporated into protein though translation; hence, only a small fraction of free cysteine is left in the cytosol. To test this hypothesis, I inhibited protein synthesis using Chx. Strikingly, Chx restored mTORC1 signaling activity and relieved the ISR under cystine starvation (Fig 22), suggesting that the bulk of cysteine is committed to protein biosynthesis. The drastic enhancement of mTORC1 signaling by Chx was also observed under normal conditions (Fig 22), indicating that protein synthesis is the predominant amino acid-consuming process in the cell. Thus, under both cystine-rich and cystine-limited conditions, the majority of available cysteine is shunted to the protein translation machinery to ensure the continuous synthesis of biologically essential proteins. Protein biosynthesis is thus a primary process that modulates intracellular amino acid balance and amino acid-dependent signaling pathways.

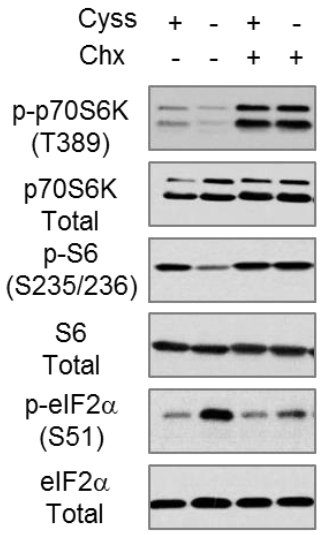


Fig 22. Inhibition of protein synthesis rescues the mTORC1 and ISR pathways during cystine limitation. HepG2 cells were pretreated with or without Chx (2.5 μM), and then treated with or without Chx (2.5 μM) in the presence or absence of cystine (100 μM) for 6 hours. Immunoblotting was performed to evaluate the levels of phosphorylated and total proteins in mTORC1 pathway and the ISR system.

4.7 Cystine limitation does not alter the integrity of mTORC1

An early study demonstrated that certain nutrients including leucine and glucose regulated the activity of mTORC1 via altering the interaction between Raptor and mTOR [7]. mTORC1 stability is also subject to redox modulation, for instance, a strong thiol-oxidizing reagent PAO dramatically decreased the amount of Raptor that bound to mTOR, with corresponding inactivation of mTORC1 [60]. Given that cysteine is a redox-active, thiol-containing amino acid, I examined whether cysteine availability has an impact on the association between mTOR and Raptor. Concordant with a previous study [60], PAO strikingly elevated phospho-p70S6K (T389) level, and co-immunoprecipitation showed that it acutely diminished the interaction between Raptor and mTOR (Fig 23). In contrast, cystine starvation did not affect the binding between Raptor and mTOR, although it consistently decreased phospho-p70S6K (T389) (Fig 23). These data suggested that distinct from the redox modulation of mTORC1 by PAO, the regulation of this complex by cystine is not though the alteration of its integrity.

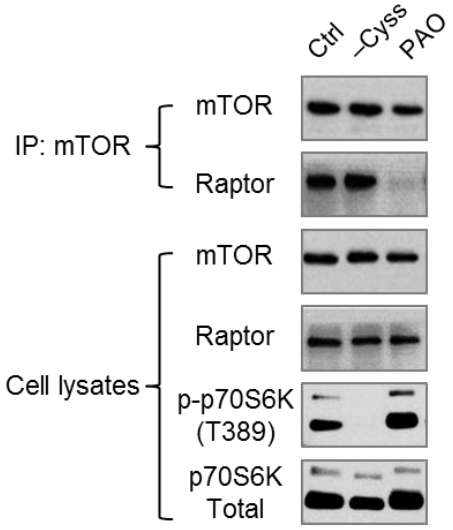
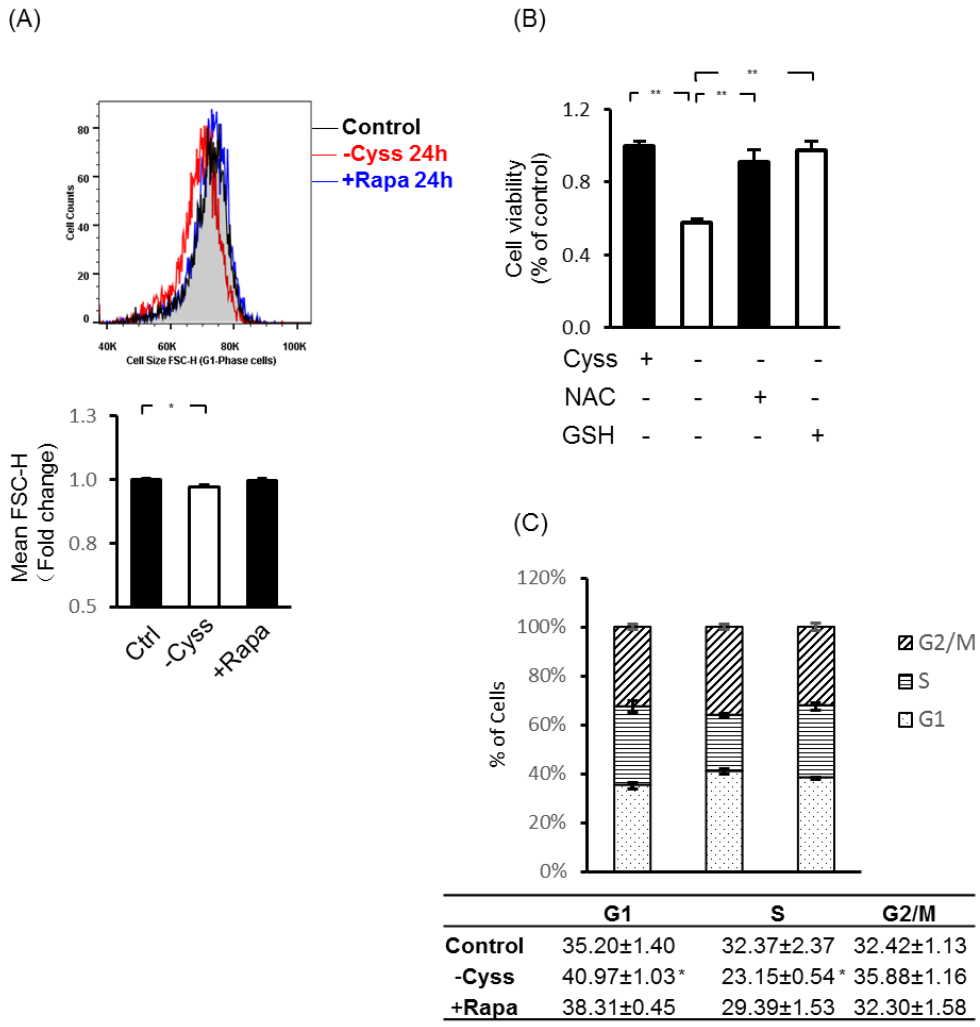


Fig 23. Cystine availability does not affect mTORC1 integrity. HepG2 cells were deprived of cystine for 9 hours, or treated with PAO (5 μM) for 15 minutes. Immunoprecipitation was performed using anti-mTOR antibody. Immunoblotting was conducted to analyze the levels of mTOR and Raptor in the immunoprecipitates (IP:mTOR), and the levels of mTOR, Raptor, phospho-p70S6K (T389) and p70S6K in the whole cell lysates (Cell lysates).

4.8 Cystine limitation reduces cell size and inhibits cell proliferation

Cell size and proliferation are two critical biological processes which are controlled by mTORC1 [80-82]. Cystine starvation for 24 hours led to a modest reduction in cell size, as indicated by the decrease in the mean FSC-H of cell population under cystine deprivation, while mTORC1 inhibition with rapamycin (20nM) did not induce any appreciable change in cell size (Fig 24A). MTT assay, which measured cell metabolic activity as a proxy of cell number and cell viability, showed that cystine starvation decreased the rate of cell proliferation (no dead cells were observed, see Fig 25A), and this inhibition was rescued by the supplementation of NAC and GSH (Fig 24B). Assessment of cell cycle distribution revealed that cystine starvation resulted in a reduction in S cell population, coupled with an increase in G1 cell population (Fig 24C), indicating that the lack of cystine impeded cell cycle progression through G1 phases. In comparison, rapamycin did not affect the distribution of cells across different phases (Fig 24C). Therefore, cystine limitation impedes cell growth and proliferation.



	G1	S	G2/M
Control	35.20±1.40	32.37±2.37	32.42±1.13
-Cyss	40.97±1.03*	23.15±0.54*	35.88±1.16
+Rapa	38.31±0.45	29.39±1.53	32.30±1.58

Fig 24. Cystine limitation inhibits cell growth and cell proliferation. (A) HepG2 cells were deprived of cystine, or treated with Rapa (20 nM) for 24 hours. Flow cytometry was performed to analyze cell size using PI/RNase. The representative histogram showing the cell size distribution (FSC-H) of G1 phase cells (upper panel) and the bar graph showing the mean FSC-H of G1 cells (lower panel) were presented. Data are the mean±SEM for n = 3 samples. *P< 0.05. (B) HepG2 were treated with or without cystine (100 μM), supplemented with or without NAC (5 mM) or GSH (1 mM) for 48 hours. MTT assay was performed. Data are the mean±SEM for n = 4 samples. **P< 0.01. (C) HepG2 cells were deprived of cystine, or treated with Rapa (20 nM) for 24 hours. Flow cytometry was performed to analyze cell cycle using PI/RNase. Cell cycle distribution was shown in the bar graph, and expressed as mean±SD for n = 3 samples in the table. *indicates that there is significant difference (P< 0.05) compared to control.

4.9 GSH protects against ferroptotic cell death during cystine limitation

Previous studies reported that the depletion of intracellular cysteine by either pharmacological means [286] or the removal of its supply [287] led to cell death through ferroptosis. Ferroptosis is a special type of cell death caused by an overwhelming degree of oxidative stress, especially lipid peroxidation, and it can be rescued by ferrostatin-1 (Fer-1) [286, 287]. I evaluated the effect of prolonged cystine deprivation on cell viability in HepG2 cells using PI staining as well as MTT. In contrast to the reported cell death in MEFs after cystine deprivation [287], HepG2 cells maintained their viability under long-term cystine limitation as evidenced by unaltered PI staining in cells (Fig 25A), although MTT reading was reduced which indicated diminished cell proliferation (Fig 25B).

Our previous data demonstrated that endogenous GSH plays protective roles under cystine starvation (Fig 20D). I therefore determined whether GSH protects HepG2 cells from death under cystine deprivation. Indeed, when intracellular GSH was depleted using BSO, dramatic cell death was detected under prolonged cystine starvation (Fig 25A and 25B). Under this condition, cell death could be rescued by Fer-1 (Fig 25A and 25B), suggesting that cells underwent ferroptosis. Notably, BSO on its own did not impair cell viability under cystine-rich conditions (Fig 25A and 25B). Furthermore, cellular lipid peroxidation was increased by BSO in the absence of cystine, and it was preventable by Fer-1 (Fig 25C), again demonstrating that it was ferroptotic cell death. In comparison, neither cystine deprivation nor GSH depletion on its own increased lipid ROS (Fig 25C), indicating that they function in parallel as defence against oxidative stress. These results indicated that cells are able to survive either cystine deprivation or GSH deletion, but not both. To further validate this point, I increased mitochondrial ROS production using antimycin A (AMA) [317], as an alternative means to deplete intracellular GSH. Likewise, AMA treatment resulted in cell death in the absence but not in the presence of cystine, and the type of cell death was ferroptosis

based on its rescue by Fer-1 (Fig 25D). Collectively, cysteine and GSH cooperate and function complementarily to maintain redox homeostasis and cell viability.

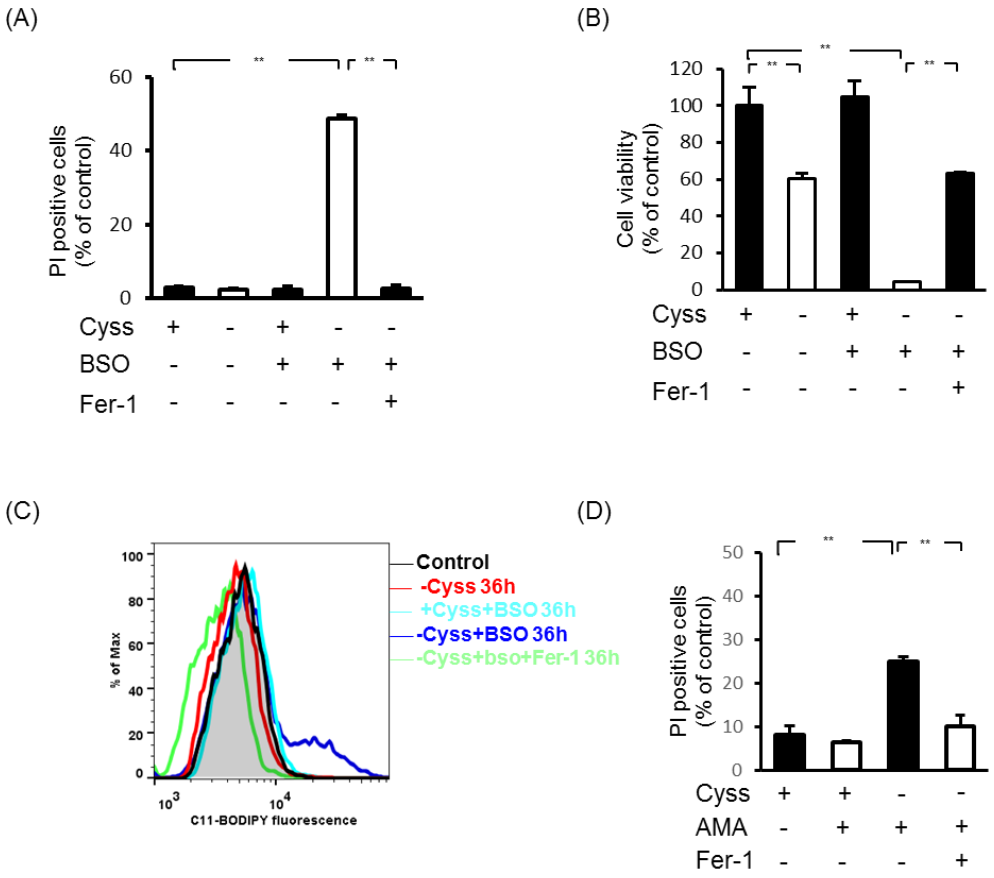


Fig 25. Endogenous GSH protects the cell against ferroptosis during cystine deprivation. (A, B) HepG2 cells were treated in DMEM supplemented with 10% dialyzed FBS with or without BSO (300 μM) in the presence or absence of cystine (100 μM) for 48 hours. Fer-1 (1 μM) was added to the indicated groups. Dead cells were detected by flow cytometry using PI staining (n=3, A), and cell viability was analyzed by MTT assay (n=6, B). Data are the mean±SEM. **P< 0.01. (C) HepG2 were treated in phenol red-free DMEM supplemented with 10% dialyzed FBS with or without BSO (300 μM) in the presence or absence of cystine (100 μM) for 36 hours. Fer-1 (1 μM) was added to the indicated groups. Lipid ROS were assessed by flow cytometry using BODIPY 581/591 C11. Representative result for 3 independent experiments is shown. (D) HepG2 cells were treated with or without AMA (20 μM) in the presence or absence of cystine (100 μM) for 48 hours. Fer-1 (1 μM) was added to the indicated groups. Dead cells were detected by flow cytometry using PI staining. Data are the mean±SEM for n=3 samples. **P< 0.01.

5. Discussion

5.1 Cysteine is essential for mTORC1 signaling and the prevention of the ISR

At present cysteine is categorized as a non-essential amino acid based on the presence of the transsulfuration pathway in the liver and other organs [243, 244]. However, this does not negate the need for exogenous uptake. This is because the transsulfuration process irreversibly consumes methionine, an essential amino acid, and adequate cysteine supplementation spares the consumption of methionine for other critical processes [249]. In addition, sufficient cysteine can ensue the production of cysteine-derived bioactive molecules such as taurine and GSH. Moreover, the transsulfuration pathway is defective in certain developmental stages and under certain pathological status [249]. The importance of cysteine has been reflected in a variety of cysteine deficiency-related diseases, which share common symptoms such as muscle wasting and immune disorder [295], and its indispensability for growth and survival has been reported in mice [269]. Besides, dietary intake of cystine has been shown to affect multiple aspects of metabolism [294]. Therefore, intracellular cysteine abundance may impose a great influence on cell biology.

mTORC1 signaling is one prominent pro-anabolic pathway which closely regulates metabolism and cell growth [62]. It is well-known to be amino acid dependent, but this list of mTORC1-stimulating amino acids mainly include essential amino acids [1, 2, 28-30, 32] and glutamine [33-36]. It is unclear whether cysteine, as a non-essential amino acid, is required for the signaling activity of this complex. Moreover, the ISR is a stress-specific adaptive pathway that reprograms multiple cellular processes [100, 102]. A previous study described an ISR target-enriched transcriptome reprogrammed by cystine starvation [318]; herein I validated this response at the signaling level and sought to decide the nature of stress.

Our results showed in HepG2 cells, cysteine is required for mTORC1 signaling and the prevention of the ISR. Removal of cystine progressively suppressed mTORC1

signaling and acutely induced the ISR (Fig 17A). The inhibition of mTORC1 signaling was coupled with the induction of autophagy, but not the activation of ERK (Fig 17A), indicating a relatively specific response of mTORC1 signaling to cystine deprivation. These signaling effects were reversible, and no crosstalk was observed between the mTORC1 and ISR pathways (Fig 17B). The absence of crosstalk between mTORC1 and the ISR in HepG2 cells is in contrast with a previous report on their signaling communication in other cell lines [123] and in my first study (Fig 12D). This discrepancy is likely due to the dose or type of mTORC1 inhibitors used and the difference in cell models. The dependence of mTORC1 signaling on cysteine also exists in another hepatoma cell line Huh 7 (Fig 17C). Moreover, NAC, a universally-used clinical supplement of cysteine, also displayed efficient mTORC1-activating property (Fig 17D). Methionine was not responsible for the signaling alteration during cystine restriction (Fig 17E), which is in agreement with the defect of transsulfuration in HepG2 cells [29]. Interestingly, the deprivation of methionine as an EAA did not induce the ISR but that of cystine did (Fig 17E), suggesting that cysteine is more "essential" than methionine for the ISR system. It is plausible that both mTORC1 and the ISR tightly integrate the signal of cysteine with cellular processes such as protein synthesis, to avoid the futile cycle of protein translation and unnecessary consumption of energy and materials when cysteine is deficient. Altogether, the limitation of cystine triggers an aberrant an anti-anabolic signaling and disrupts cellular homeostasis.

5.2 Cysteine is the primary determinant of GSH homeostasis

The biological significance of cysteine goes beyond its role as a basic building block for proteins, and of particular importance is its role as the rate-limiting precursor for the biosynthesis of GSH [244]. As expected, the removal of cystine supply gradually decreased intracellular GSH level, and this was correlated with an increase in its export (Fig 18A). The elevated GSH export was reminiscent of a cellular adaptive response to cystine limitation, by enhancing the flux of GSH through the γ-glutamyl cycle in a

bid to recycle cysteine. In line with this notion, inhibition of GSH ecto-degradation unmasked the accumulation of GSH at the extracellular milieu during the first few hours of cystine deprivation (Fig 18B), which also suggested that a large proportion of exported GSH undergoes degradation. Cystine availability is exclusively accountable for GSH homeostasis, as the supplementation of cystine alone restored GSH metabolism to the normal state even when other amino acids were unavailable (Fig 18C). Thus, under physiological conditions, insufficient cystine intake would disrupt hepatic GSH homeostasis, which has been confirmed by *in vivo* studies [260, 261]. Cystine limitation compromises intracellular GSH pool not only by restricting its *de novo* synthesis, but also driving its export and ecto-degradation which further drains intracellular GSH stores. The sacrifice of intracellular GSH content to maintain cysteine level is a reflection of the critical function of GSH as a cysteine reservoir.

5.3 Cystine supply is required to maintain amino acid balance but dispensable for redox balance and proteostasis

Surprisingly, although GSH is deemed as the major antioxidant defense against ROS [244, 257], oxidative stress did not arise under cystine deprivation (Fig 19A, 19B, and 19C) even when intracellular GSH content was compromised (Fig 18A). In HepG2 cells, cystine starvation increased neither soluble ROS (Fig 18A) nor lipid ROS (Fig 19B and 19C). These observations were consistent with the report that prolonged cystine starvation did not alter the transcription of genes involved in oxidative stress response signaling in HepG2/C3A cells [318]. It suggested that the remaining reducing force of GSH or alternative mechanism is at play to guard against oxidative stress during cystine limitation. In comparison, the xCT antagonist SAS elevated both soluble and lipid ROS (Fig 19A, 19B, and 19C), and it suggested that this drug elicits a change cellular redox different from that by cystine deprivation, which was likely resulted from unspecific effects unrelated to xCT inhibition [319].

GSH/GSSG exists as important redox buffer in the ER which likely participates in disulfide bond formation and reduction of non-native disulfide bond in the ER, and the shortage of GSH led to abnormal disulfide linkage [310]. However, in HepG2 cells, cystine deprivation, although resulting in a reduction of intracellular GSH (Fig 18A), did not cause ER stress as evidence by the absence of its markers including PERK phosphorylation and Bip, which were otherwise induced by the ER inducer Tm (Fig 19D). In comparison to PERK, cystine deprivation selectively activated another upstream kinase in the ISR system, GCN2 (Fig 19D). GCN2 specifically senses the imbalance in intracellular amino acids and initiates AAR through eIF2α-ATF4 [102]. Therefore, these results suggested that while the supply of cystine is dispensable for redox balance in the cytosol and proteostasis in the ER, it is indispensable for sustaining amino acid balance. The ISR system is sensitive to the restriction of cystine, which specifically triggers the AAR signaling in this network.

5.4 Supplemented GSH sustains mTORC1 signaling and suppresses the ISR during cystine deprivation through its release of cysteine

GSH has been highlighted by numerous studies as a vital antioxidant that impinges on a variety of cellular processes [257]. In spite of its antioxidant potential, our data demonstrated that GSH per se has no regulatory effect on either mTOR signaling or the ISR; instead, GSH modulates these two pathways through the secondary production of cysteine. First, although supplementation with high amount of exogenous GSH was able to rescue mTORC1 signaling activity and relieve the ISR under cystine limitation, these reversal effects no longer existed when GSH degradation was inhibited by OU749 (Fig 20A). Second, the membrane-permeable GSHee restored mTORC1 signaling and reduced the ISR during cystine limitation only when GGT was active (Fig 20B). Compared to GSH, GSHee partially sustained mTORC1 under cystine deprivation (Fig 20B), which could be attributed to the fact that GSHee readily enters the cell and thus tends not to accumulate in the extracellular milieu as a GGT substrate.

Third, the drastic depletion of GSH by BSO had no appreciable effect on mTORC1 and the ISR, whereas it worsened the negative effect of cystine limitation on mTORC1 signaling (Fig 20C and 20D). Our results draw attention to the cysteine-replenishing role of GSH, rather than its antioxidant property, as a critical mechanism mediating its pro-anabolic, stress-resistant functions under adverse conditions. This mechanism is at least partially accountable for the pro-survival role of GSH in cancers, in which the highly abundant GSH continuously provides cysteine to support cancer growth in the nutrient-limited microenvironment [320]. GGT is the key enzyme in this cysteine supply chain. Concordantly, GGT is expressed at high levels over the entire surface of cancer cells [321], which serves the purpose of maximizing the recycling of cysteine from GSH. Hence, the role of GSH as a cysteine reservoir is equally important, if not more crucial, to its role as an antioxidant defense.

5.5 GSH facilitates in the maintenance of a normal amino acid metabolic program during cystine limitation

Quantitative analysis of mRNA expression showed that the restriction of cystine upregulated the transcription of AARE-containing genes, including those involved in amino acid metabolism (*SLC7A11*, *SLC7A1*, and *SLC38A2*) and cell survival (*TRIB3*) (Fig 21). Consistently, microarray study also demonstrated that the majority of differentially expressed genes upon cystine deprivation were downstream of the ISR system, and most of them clustered in the AAR [318]. These data provided additional evidence that cystine starvation induced a stress of amino acid limitation by nature (Fig 19D and 21). These transcriptional changes could be prevented by the supplementation of GSH, but the inhibition of GGT abolished the preventive effect (Fig 21), consistent with the signaling regulation of the ISR by GSH (Fig 20A). Thus, cystine limitation transcriptionally reprograms those ISR genes especially those involved in amino acid metabolism, and GSH facilitates in restoring the transcriptional program to the normal state during cystine restriction.

5.6 The majority of available cysteine is committed to protein biosynthesis under cystine deprivation

Protein biosynthesis appears to take precedence over other cysteine-consuming processes, given that cysteine is preferentially shunted to the protein synthetic pathway under basal conditions in general and during sulfur restriction in particular [316]. This is ensured at the biochemical level: the Km of L-cysteinyl-tRNA synthetase towards cysteine, which directs cysteine to the translational machinery, is less than one tenth of that for other enzymes in cysteine metabolism such as GCS or cysteine dioxygenase [316]. Consistent with this view, our data demonstrated that a large proportion of intracellular cysteine is used for protein synthesis under cystine deprivation (Fig 22). Inhibition of protein synthesis was sufficient to restore mTORC1 signaling and relieve the ISR during cystine limitation (Fig 22). Hence, even though it is being attenuated, protein synthesis remains to be the predominant process that consumes cysteine under cystine starvation; its enormous consumption of cysteine may provide an explanation why endogenous GSH is unable to completely prevent the decline in intracellular free cysteine and the subsequent suppression of mTORC1 signaling in cystine-limited conditions (Fig 20D). The blockage of protein synthesis also activated mTORC1 under normal conditions (Fig 22), suggesting that the activity of protein synthesis has a great influence on this amino acid signaling pathway. Protein synthesis, as an energy-consuming and redox-relevant process, was linked to stress and the impairment of cell viability by some studies [99, 101, 322]. Based on our results, protein biosynthesis is a principal cellular process that determines amino acid homeostasis and modulates amino acid-sensitive signaling networks in the cell.

5.7 Cystine starvation does not affect mTORC1 stability

The kinase activity of mTORC1 requires Raptor, which creates docking sites for mTOR substrates such as p70S6K and 4E-BP1, allowing the phosphorylation reaction to occur [323]. A study showed that Raptor participates in the sensing of nutrient and

stress, and its interaction with mTOR is altered in response to various stimulus including leucine availability and glucose deprivation [7]. Particularly, the interaction between Raptor and mTOR is highly sensitive to thiol oxidants such as PAO [60]. However, in contrast to the reduction in the binding of Raptor to mTOR by PAO, cystine starvation did not affect the interaction between Raptor and mTOR (Fig 23). This result indicated that the limitation of cystine may regulate mTORC1 in a different manner as that by leucine, and it is unlikely to regulate this complex in a redox-dependent way. The precise mechanism by which cysteine regulates mTORC1 remains to be uncovered.

5.8 Cystine starvation exerts anti-growth and anti-proliferative effects

mTORC1 signaling controls cell size accumulation [80] and cell proliferation [81, 82], and dysregulated mTORC1 signaling is associated with diseases such as cancer and skeletal muscle atrophy [91]. In line with the suppression of mTORC1 signaling, cell size was reduced and cell proliferation was inhibited under cystine starvation, by a greater extent than that under rapamycin treatment (Fig 24A and 24B), and cell proliferation could be restored by GSH and NAC (Fig 24B). Examination of different phases of cell cycle suggested that cystine restriction retards the progression of cell cycle through G1 phases (Fig 24C). Thus, adequate cystine uptake is required for normal cell growth and cell proliferation, which is consistent with the cysteine deficiency-associated physiological phenotypes such as growth retardation in mice [269] and muscle wasting in humans [295].

5.9 Intracellular cysteine and GSH cooperate to maintain redox balance and cell viability

Cystine starvation was shown to be lethal in certain cellular and experimental contexts, inducing ferroptosis [286, 287]. However, the hepatoma cells HepG2 were resistant to cystine deprivation-induced cell death (Fig 25A and 25B). In addition, HepG2 could also withstand the drastic depletion of GSH by BSO (Fig 25A and 25B). Moreover,

neither cystine limitation nor GSH depletion elicited obvious oxidative stress based on the lack of lipid peroxidation (Fig 25C). Strikingly, when cystine and GSH were depleted simultaneously, cell viability was dramatically compromised (Fig 25A and 25B), coupled with a burst of lipid ROS (Fig 25C). These cells died of ferroptosis in this scenario based on its rescue by Fer-1 (Fig 25A and 25B). Likewise, ferroptosis was induced by AMA under cystine starvation (Fig 25D), which can also deplete intracellular GSH. These results demonstrated that the presence of either cysteine or GSH, even if GSH is at subnormal level, is sufficient to guard against oxidative stress and ferroptotic cell death in HepG2 cells. Reports showed that either NAC supplementation [270] or xCT overexpression [324] was able to preserve the viability of GCLC knockout cells, and GCLM null tumour cells compensatorily upregulated cystine uptake to promote survival [279]. Additionally, cysteine/cystine is tentatively considered as a cellular redox couple that functions in parallel with GSH/GSSG [254, 325]. Therefore, cysteine and GSH likely exist as redundant reducing powers, and they complement each other in maintaining redox balance and cell viability. In addition, the dual depletion of cysteine and GSH is a promising strategy to kill resilient cancer cells.

5.10 Difference in mTORC1 dynamics under the deprivation of total amino acid or cystine observed in the two studies

Comparing the results of these two projects, an interesting but elusive issue is that mTORC1 signaling responds in differential ways during prolonged total amino acid starvation and under cystine deprivation. Based on the first project, in C2C12 mouse myotubes, under the deprivation of total amino acids (except for glutamine), autophagy is mobilized and it generates sufficient amino acids to restore mTORC1 signaling. However, in HepG2 cells, during cystine limitation, mTORC1 signaling declines progressively without being recovered, although autophagy is also activated in this setting. A few thoughts are proposed here in an attempt to reconcile this discrepancy of mTORC1 dynamics in these two contexts: (1) The functional transsulfuration

pathway in C2C12 myotubes mediates the conversion of autophagy-derived methionine to cysteine and therefore enables the reactivation of mTORC1 upon prolonged amino acid starvation. However, this compensatory mechanism is absent in HepG2 cells under cystine deprivation. (2) It is possible that differential autophagic degradation programs are induced under total amino acid starvation in mouse myotubes versus under cystine restriction in HepG2 cells, which determines the feedback on mTORC1 signaling. This may include differences in degradation targets and magnitude of autophagic flux. In addition, essential proteins that contain cysteine in their functional modules may be spared from degradation given their biological importance, so only a limited portion of cysteine is released by degradation under cystine limitation. (3) The continuous or even enhanced incorporation of cysteine into cysteine-rich proteins drives the decline in intracellular free cysteine under cystine starvation in HepG2 cells. A few cysteine-rich proteins, such as those encoding metallothionein (MT1E and MT1H), were upregulated at the transcriptional level under cystine starvation [318], and the translation of these mRNAs requires the incorporation of cysteine. However, this adaptive response may not take place under amino acid starvation in mouse myotubes. These proposed explanations remain largely speculative, but they are interesting possibilities to explore, which can reveal cell context and stress-dependent differences in the autophagic program and amino acid signaling. If possible, these works will be pursued in the future as an extension to the current studies.

6. Concluding remarks for the second study

This study highlighted the critical role of cysteine for anabolic signaling and cell homeostasis, and the crosstalk between cystine and GSH in the modulation of cell signaling network (the model proposed based this study is shown in Fig 26). In hepatoma cells, cystine deprivation inhibits the mTORC1 pathway, induces the ISR, and curbs cell growth and proliferation. GSH counters these stresses through supplying cysteine via the γ-glutamyl cycle during cystine limitation, which is unrelated to its

antioxidant potential. Moreover, GSH protects cells from oxidative challenge and ferroptosis under cystine deprivation; in fact, GSH and cysteine act redundantly and complementarily to defend against oxidative stress and cell death. The attenuation of mTORC1 signaling and the perturbation of the ISR system likely underlie cysteine deficiency-implicated pathological circumstances.

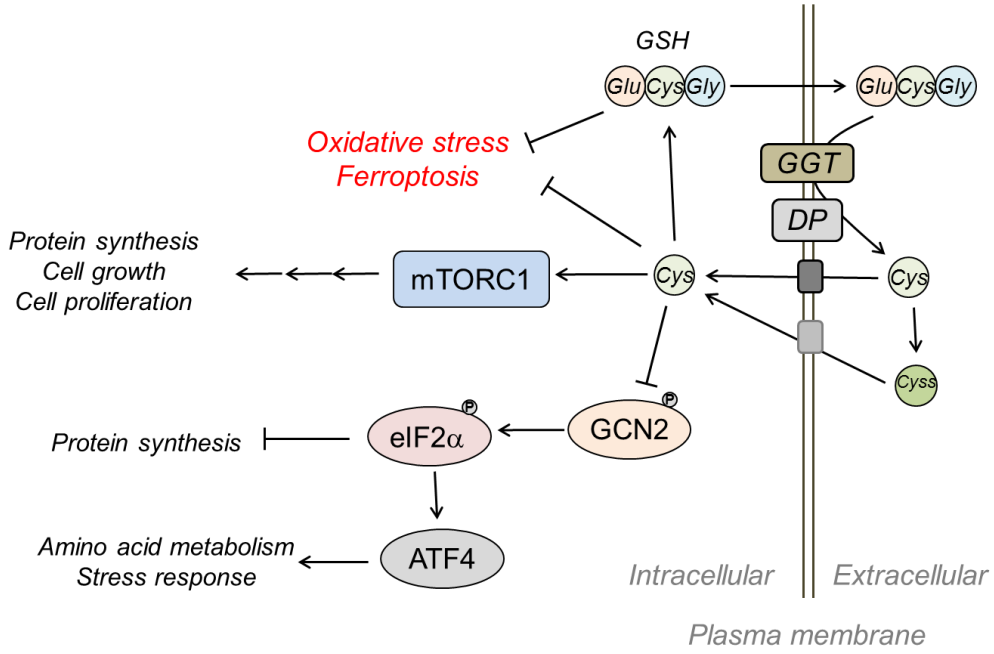


Fig 26. Schematic diagram of the model showing the cooperation between cystine and GSH in the regulation of amino acid signaling pathways and cellular processes. In HepG2 cells, abundant intracellular cysteine is required for mTORC1 signaling and the prevention of the ISR, which are closely related to protein synthesis and other essential cellular processes. GSH indirectly regulates mTORC1 and the ISR by providing cysteine through the γ-glutamyl cycle. What's more, cellular cysteine and GSH cooperate to guard against oxidative stress and the induction of ferroptosis. This paradigm highlights the close inter-relationship between cysteine and GSH and their complementary functions in the modulation of stress resistance, cell viability and cell growth.

General Conclusions and Perspectives

Amino acids are critical for cell survival since they serve as substrates for protein synthesis and metabolism as well as signaling cues that modulate signaling pathways. During amino acid starvation, the cells mobilize internal nutrient stores and initiate adaptive responses. The mTORC1 pathway and the ISR are two major amino acid signaling pathways in the cell which sense amino acid signals and impose reprogramming on multiple processes such as protein synthesis, metabolism and cell growth.

Skeletal muscle is a metabolically flexible tissue that can withstand modest nutritional stress, and autophagy is a critical intrinsic mediator of stress resistance in this system. In cultured mouse myotubes, autophagy serves as an affluent amino acid source, and it generates relative sufficient amino acids to sustain mTORC1 signaling during general amino acid limitation. This feedback from autophagy to mTORC1 is important for continuous protein synthesis and other anabolic processes as well as cell growth. Autophagy guards against stress and prevents the agitation of the ISR system, which is presumably attributed to its housekeeping role rather than its role as a nutrient store. The defect in autophagy would conceivably undermine the plasticity of skeletal muscle under nutrient limitation and result in dyshomeostasis, eventually impairing skeletal muscle function.

However, not every amino acid is the same and specific cellular programs are initiated under the lack of individual amino acids. This is especially the case under cystine limitation. Cysteine is a unique thiol-containing amino acid and it is closely related to the key antioxidant GSH. In human hepatoma HepG2 cells, this non-essential amino acid is indispensable for mTORC1 signaling and cellular homeostasis. Cystine limitation suppresses mTORC1 signaling, induces the ISR, and hinders cell growth and proliferation. Under this condition, GSH performs as the protective force against these stresses, which is mediated by the release of cysteine via its export and ecto-

degradation rather than its antioxidant potential. In addition, cellular cysteine and GSH exist as redundant and complementary defences against oxidative stress and ferroptosis. The role of GSH as a cysteine reservoir is therefore worthy of attention which is critical for cell survival and growth. Cysteine is an unforgettable “essential” amino acid for anabolic signaling, and it may fulfil redox roles in parallel with GSH.

Several outstanding questions arising from the present studies require further exploration. For instance, why do total amino acid and cystine limitation result in differential signaling changes in terms of mTOR1 signaling and the ISR? Why does autophagy fail to maintain mTORC1 signaling under cystine deprivation in HepG2 cells? What is the mechanism by which autophagy mitigates the ISR? In addition, what is the cysteine-sensing mechanism for the mTORC1 pathway? Researches that address these questions will provide a clearer picture of the context-specific cellular response under certain conditions of amino acid limitation or imbalance.

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